

## PCT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

DUBUC, Jean, H.  
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The Stock Exchange Tower  
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800 Place Victoria  
Montreal, Quebec H4Z 1E9  
CANADA

Date of mailing (day/month/year)

20 June 2000 (20.06.00)

Applicant's or agent's file reference

AML/10857.274

International application No.

PCT/CA99/00987

International filing date (day/month/year)

21 October 1999 (21.10.99)

## IMPORTANT NOTIFICATION

1. The following indications appeared on record concerning:

☐

the applicant

☐

the inventor

☒

the agent

☐

the common representative

Name and Address

DUBUC, Jean, H.  
Goudreau Gage Dubuc & Martineau  
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State of Residence

Telephone No.

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Facsimile No.

514 397 4382

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐

the person

☐

the name

☒

the address

☐

the nationality

☐

the residence

Name and Address

DUBUC, Jean, H.  
Goudreau Gage Dubuc  
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Suite 3400, P.O. Box 242  
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Montreal, Quebec H4Z 1E9  
Canada

State of Nationality

State of Residence

Telephone No.

514 397 7675

Facsimile No.

514 397 4382

Teleprinter No.

3. Further observations, if necessary:

**The new agent's address on the Demand has been considered as a change under Rule 92bis. In case of disagreement, the International Bureau should be notified immediately.**

4. A copy of this notification has been sent to:

☒

the receiving Office

☐

the designated Offices concerned

☐

the International Searching Authority

☒

the elected Offices concerned

☒

the International Preliminary Examining Authority

☐

other:

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

C. Cupello

Telephone No.: (41-22) 338.83.38

## PCT COOPERATION TREATY

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## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
 United States Patent and Trademark  
 Office  
 Box PCT  
 Washington, D.C.20231  
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 20 June 2000 (20.06.00)	
<b>International application No.</b> PCT/CA99/00987	<b>Applicant's or agent's file reference</b> AML/10857.274
<b>International filing date (day/month/year)</b> 21 October 1999 (21.10.99)	<b>Priority date (day/month/year)</b> 21 October 1998 (21.10.98)
<b>Applicant</b> BEAUDOIN, Adrien et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

16 May 2000 (16.05.00)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
 34, chemin des Colombettes  
 1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

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## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

#6 attachment  
09/830/46

Applicant's or agent's file reference <b>AML/10857.274</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/CA 99/ 00987</b>	International filing date (day/month/year) <b>21/10/1999</b>	(Earliest) Priority Date (day/month/year) <b>21/10/1998</b>
Applicant <b>UNIVERSITE DE SHERBROOKE et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

**4. With regard to the title,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

**5. With regard to the abstract,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

**6. The figure of the drawings to be published with the abstract is Figure No.**

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00987

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C11B1/10 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C11B C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 12, 26 December 1996 (1996-12-26) - & JP 08 198754 A (YAKULT HONSHA CO LTD) ✓ 6 August 1996 (1996-08-06) abstract	1, 2, 5, 14, 15, 18, 20
Y	---	27
Y	WO 84 01715 A (HELLGREN LARS G I ; MOHR VIGGO (NO); VINCENT JAN GUSTAV (SE)) 10 May 1984 (1984-05-10) page 7, line 29 - line 31 examples 1, 2 --- -/-	27



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

31 January 2000

Date of mailing of the international search report

28/02/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Authorized officer

Dekeirel, M

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00987

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI  Section Ch, Week 197845  Derwent Publications Ltd., London, GB;  Class C03, AN 1978-80681A  XP002129262  -&amp; JP 53 112195 A (NIPPON PAINT CO LTD),  30 September 1978 (1978-09-30)  abstract</p>	15
X	<p>PATENT ABSTRACTS OF JAPAN  vol. 009, no. 160 (C-289),  4 July 1985 (1985-07-04)  -&amp; JP 60 035057 A (SANEI KAGAKU KOGYO KK),  22 February 1985 (1985-02-22)  abstract</p>	15, 16, 25
X	<p>PATENT ABSTRACTS OF JAPAN  vol. 016, no. 249 (C-0948),  8 June 1992 (1992-06-08)  -&amp; JP 04 057853 A (CHLORINE ENG CORP  LTD; OTHERS: 01),  25 February 1992 (1992-02-25)  abstract</p>	25
Y		26
Y	<p>EP 0 732 378 A (NIPPON OIL CO LTD) ✓  18 September 1996 (1996-09-18)  column 1, line 37 - line 47  column 4, line 49 - line 53</p>	26
X	<p>DATABASE WPI  Section Ch, Week 197633  Derwent Publications Ltd., London, GB;  Class D13, AN 1976-62648X  XP002129263  -&amp; JP 51 076467 A (KYOWA HAKKO KOGYO), ✓  2 July 1976 (1976-07-02)  abstract</p>	25, 26
X	<p>CHEMICAL ABSTRACTS, vol. 98, no. 21,  23 May 1983 (1983-05-23)  Columbus, Ohio, US;  abstract no. 177859,  RAA, JAN ET AL.: "Isolation of  astaxanthin from crayfish or shrimp waste  for use as a coloring agent in fish feed"  page 531; column 2;  XP002129261  abstract  -&amp; NO 147 365 B ✓  20 December 1982 (1982-12-20)</p> <p style="text-align: center;">-/--</p>	25

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00987

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PATENT ABSTRACTS OF JAPAN vol. 009, no. 059 (C-270), 15 March 1985 (1985-03-15) ✓ - &amp; JP 59 196032 A (FUMIO NISHIKAWA; OTHERS: 02), 7 November 1984 (1984-11-07) abstract</p> <p>-----</p>	27

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 99/00987

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 08198754 A	06-08-1996	NONE	
WO 8401715 A	10-05-1984	AT 42111 T AU 573730 B AU 2204183 A BR 8307587 A CA 1220740 A DK 308684 A EP 0107634 A FI 842555 A,B, JP 1934186 C JP 5262665 A JP 6062994 B JP 5017209 B JP 59501908 T NO 842497 A US 4963491 A ZA 8307888 A SE 8302268 A US 4801451 A	15-04-1989 23-06-1988 22-05-1984 25-09-1984 21-04-1987 22-06-1984 02-05-1984 25-06-1984 26-05-1995 12-10-1993 17-08-1994 08-03-1993 15-11-1984 21-06-1984 16-10-1990 26-09-1984 23-10-1984 31-01-1989
JP 53112195 A	30-09-1978	NONE	
JP 60035057 A	22-02-1985	JP 1383716 C JP 61052183 B	09-06-1987 12-11-1986
JP 04057853 A	25-02-1992	JP 2963152 B	12-10-1999
EP 0732378 A	18-09-1996	JP 8253695 A CA 2171780 A NO 961074 A	01-10-1996 17-09-1996 17-09-1996
JP 51076467 A	02-07-1976	JP 1000118 C JP 52048191 B	30-05-1980 08-12-1977
NO 147365 B	28-05-1982	NO 803582 A,B, DK 515581 A	28-05-1982 28-05-1982
JP 59196032 A	07-11-1984	NONE	

## NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

### "Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international application is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

### Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

### Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C11B 1/10, C12N 9/64</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/23546</b> <b>(43) International Publication Date:</b> 27 April 2000 (27.04.00)
<b>(21) International Application Number:</b> PCT/CA99/00987 <b>(22) International Filing Date:</b> 21 October 1999 (21.10.99)  <b>(30) Priority Data:</b> 2,251,265 21 October 1998 (21.10.98) CA  <b>(71) Applicant (for all designated States except US):</b> UNIVERSITE DE SHERBROOKE [CA/CA]; University Boulevard, Sherbrooke, Quebec J1K 2R1 (CA).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BEAUDOIN, Adrien [CA/CA]; 748, boulevard des Vétérans, Rock Forest, Quebec J1N 1Z7 (CA). MARTIN, Geneviève [CA/CA]; 797, McManamy, Sherbrooke, Quebec J1H 2N1 (CA).  <b>(74) Agents:</b> DUBUC, Jean, H. et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, P.O. Box 242, 800 Place Victoria, Montreal, Quebec H4Z 1E9 (CA).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES  <b>(57) Abstract</b>  Provided herein is a method for extracting lipid fractions from marine and aquatic animal material by acetone extraction. The resulting non-soluble and particulate fraction is preferably subjected to an additional solvent extraction with an alcohol, preferably ethanol, isopropanol or <i>t</i> -butanol or an ester of acetic acid, preferably ethyl acetate to achieve extraction of the remaining soluble lipid fraction from the marine and aquatic animal material. The remaining non-soluble particulate contents is also recovered since it is enriched in proteins and contains a useful amount of active enzymes. Also provided herein is a krill extract.		

**FOR THE PURPOSES OF INFORMATION ONLY**

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## METHOD OF EXTRACTING LIPIDS FROM MARINE AND AQUATIC ANIMAL TISSUES

### BACKGROUND OF THE INVENTION

5 This invention relates to the extraction of lipid fractions from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals. More specifically, this invention relates to an improved method of extracting lipid fractions by dehydration with solvents and recovering a solid residue rich in active enzymes.

10 Lipid fractions obtained from marine and aquatic animals such as krill, *Calanus*, fish and sea mammals have various applications:

#### Medical applications

15 Marine and aquatic animal oils and fractions thereof contain various therapeutic agents. For example, it is reported that various marine and aquatic animal oils have anti-inflammatory properties. Marine and aquatic animal oils are also reported as helpful in reducing the incidence of cardiovascular disease. Also, some marine and aquatic animal oils are reported as suppressing the development of certain forms of lupus and renal diseases. As a further example, krill may be used as a source of enzymes for debridement of ulcers and wounds or to facilitate food digestion. Also  
20 marine and aquatic oils contain various antioxidants, which may have potential therapeutic properties.

#### Nutraceuticals

25 Considering the beneficial effects of omega-3 fatty acids, oils from krill, *Calanus* and fish could be used as dietary supplements to human diet. These fatty acids are essential for proper development of the brain and the eye. Marine and aquatic animal oils are also rich in liposoluble vitamins A, D and E and carotenoids.

#### Cosmetics

Various marine and aquatic animal oils are used for the production of moisturizing creams.

### Fish farming

Among the lipids found in krill, *Calanus* and fish, high concentrations of fatty acids 20:5 (eicosapentaenoic acid) and 22:6 (docosahexaenoic acid) are present. These fatty acids are essential nutrients and are beneficial as fish feed. Furthermore, these essential nutrients are carried over in human diet by eating the fish grown on such diets.

### Animal feed

Animal feed diets rich in omega-3 fatty acids may increase the level of unsaturated fatty acids and decrease cholesterol levels of meat. This property is already exploited in the poultry industry to improve the quality of eggs.

Various methods for extracting marine and aquatic animal oils are known. For example, it is known to extract fish oil using organic solvents such as hexane and ethanol. It is also known to measure the fat content in fish muscle tissue using solvents such as acetone.

USP 4,331,695 describes a method using pressurized solvents which are gaseous at room temperature, such as propane, butane or hexane. The extraction is performed at preferred temperatures of 15 to 80°C on shredded vegetable or finely divided animal products. The extracted oils are then made to precipitate under high pressure and elevated temperatures of 50 to 200°C. However, hexane is a poor extraction solvent for marine animals such as krill. Furthermore, the high temperatures used in the precipitation step negatively alters the lipids.

Canadian Patent Application 2,115,571 describes a method for extracting oils from various brown and red algae species. The method provides for example Soxhlet extraction using nearly pure ethanol for 40 hours.

USP 5,006,281 describes a method for extracting oil from marine and aquatic animals such as fish. The marine and aquatic animal is first treated with an antioxidant compound, finely divided and centrifuged to separate the oil phase from

the aqueous phase and solid phase. The oil phase is then further treated with antioxidant to remove undesirable odour or taste.

Canadian Patent 1,098,900 describes a method for extracting oils from krill. The method involves emulsifying fresh or defrosted krill in an aqueous medium. The oil fraction is recovered by centrifugation.

Folch in the article published in the year 1957 in J. biol. Chem. 226: 497-509 "*A simple method for the isolation and purification of total lipids from animal tissues*" proposes an extraction method using chloroform and methanol. This method is not commercially feasible because of the toxicity of the solvents involved.

However, prior art processes are generally commercially unfeasible or provide low quantitative yields. Thus, it is an object of the present invention to provide an improved marine and aquatic animal oil extraction method allowing recovery of a valuable lipid fraction and separate recovery of a valuable protein rich solid residue that comprises active enzymes.

Other objects and further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. It should be understood, however, that this detailed description, while indicating preferred embodiments of the invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1. Gas-liquid chromatography of fatty acids from dry krill (chloroform-methanol)

Figure 2. Gas-liquid chromatography of fatty acids from dry krill (acetone)

Figure 3. Gas-liquid chromatography of fatty acids from frozen krill (acetone)

Figure 4. Gas-liquid chromatography of fatty acids from frozen krill (ethanol)

Figure 5. Gas-liquid chromatography of fatty acids from frozen krill (*t*-butanol)

Figure 6. Gas-liquid chromatography of fatty acids from frozen krill (ethyl acetate)

Figure 7. Thin-layer chromatography of neutral lipids of *Calanus* sp. and *M. norvegica*

5 Figure 8. Thin-layer chromatography of neutral lipids of *E. pacifica*

Figure 9. Thin-layer chromatography of neutral lipids of *M. schmitti*

Figure 10. Thin-layer chromatography of neutral lipids of *G. galeus*

Figure 11. Thin-layer chromatography of neutral lipids of Angel Shark

Figure 12. Thin-layer chromatography of phospholipids of *Calanus* sp. and  
10 *M. norvegica*

Figure 13. Thin-layer chromatography of phospholipids of *E. pacifica*

Figure 14. Thin-layer chromatography of phospholipids of *M. schmitti*

Figure 15. Thin-layer chromatography of phospholipids of *G. galeus*

Figure 16. Thin-layer chromatography of phospholipids of Angel Shark

15 Figure 17. Influence of the volume of acetone on lipid extraction (*E. pacifica*)

Figure 18. Influence of incubation time in acetone on lipid extraction  
(*E. pacifica*)

Figure 19. Influence of the volume of ethanol on lipid extraction (*E. pacifica*)

Figure 20. Influence of incubation time in ethanol on lipid extraction  
20 (*T. raschii*)

#### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT**

Before describing the present invention in detail, it is to be understood that the invention is not limited in its application to the process details described herein. The  
25 invention is capable of other embodiments and of being practised in various ways. It is also to be understood that the phraseology or terminology used herein is for the purpose of description and not limitation.

The method of the invention comprises suspending freshly collected marine and  
30 aquatic material in acetone. Lipids are extracted with a ketone such as acetone. This allows a rapid dehydration of animal tissue and a migration of the lipid fraction to the solvent. The dry residue is a valuable product rich in active enzymes.

In a preferred embodiment, the extraction is carried out by successive acetone and alcohol treatments. Preferred alcohols are isopropanol, and *t*-butanol. The alcohol may also be substituted with an ester of acetic acid such as ethyl acetate. The procedure produces two successive lipid fractions and a dry residue enriched in protein, including active enzymes. Recovery of total lipids is comparable to the Folch et al. (1957) procedure reported in the background of the invention. It has been tested with krill, *Calanus*, fish and shark tissues.

Surprisingly, it was found that successive extraction treatments as proposed by the present invention has a better yield in lipid extraction than single solvent system extractions. The extraction using two successive solvents which starts with a ketone such as acetone is especially advantageous since the acetone, in effect, dehydrates the animal tissue. Having the animal tissue in dehydrated form greatly facilitates the extraction process with the second solvent, alcohol or an ester of acetic acid such as ethyl acetate.

In the case of zooplankton such as krill and *Calanus* and in the case of fish-filleting by-products such as fish viscera, it is noted that extraction with acetone alone may be sufficient to allow a cost-effective recovery of lipid fractions and separate recovery of a dry solid product rich in proteins including active enzymes.

The general extraction method of the present invention will now be described. The starting material consisting of freshly harvested and preferably finely divided marine and aquatic animal material is subjected to acetone extraction, for at about two hours and preferably overnight. However extraction time is not critical to the yield of lipid extraction. To facilitate extraction, it is preferable to use particles of less than 5mm in diameter. Extraction is preferably conducted under inert atmosphere and at a temperature in the order of about 5°C or less.

Preferably, the beginning of the extraction will be conducted under agitation for about 10 to 40 minutes, preferably 20 minutes. Although extraction time is not critical, it

was found that a 2 hour extraction with 6:1 volume ratio of acetone to marine and aquatic animal material is best.

5 The solubilized lipid fractions are separated from the solid material by standard techniques including, for example, filtration, centrifugation or sedimentation. Filtration is preferably used.

10 After separation by filtration on an organic solvent resistant filter (metal, glass or paper) the residue is optionally washed with pure acetone, preferably two volumes (original volume of material) to recover yet more lipids. The combined filtrates are evaporated under reduced pressure. Optionally, flash evaporation or spray drying may be used. The water residue obtained after evaporation is allowed to separate from the oil phase (fraction I) at low temperature.

15 The solid residue collected on the filter is suspended and extracted with alcohol, such as ethanol, isopropanol, *t*-butanol or alternatively with ethyl acetate, preferably two volumes (original volume of material). The filtrate is evaporated leaving a second fraction of lipids (identified as fraction II). Although the extraction period is not critical, it was found that an extraction time of about 30 minutes is sufficient at  
20 temperatures below about 5°C.

Temperature of the organic solvents, except *t*-butanol, and temperature of the sample are not critical parameters, but it is preferable to be as cold as possible. However, in the case of *t*-butanol which is solid at room temperature, it is important  
25 to warm it before using it and to perform the extraction at 25 °C immediately.

#### Comparative examples

30 To compare the efficiency of the extraction process, a classical technique (Folch et al. 1957) using chloroform and methanol was applied to krill. This method is the reference for measuring efficiency of the extraction process. Another comparison has been made with a technique using hexane as the extraction solvent. Lipid recovery



was estimated by suspending lipid fractions in small volumes of their original solvents and measuring by gravimetry small aliquots after evaporation.

For all examples provided herein, the method of the present invention involving acetone extraction followed by extraction with a second solvent (ethyl acetate, for example) gave a translucent oil having appearance and properties more attractive than any oil obtained by the classical technique of Folch et al. (1957).

To analyze lipid composition, 780 µg of each extract was loaded on silica-gel plates and fractionated by thin layer chromatography, TLC (Bowyer et al. 1962) with the following solvents. Neutral lipids: hexane, ethyl ether, acetic acid (90:10:1, v/v) and phospholipids: chloroform, methanol, water (80:25:2, v/v). Fatty acid composition of *E. pacifica* was analyzed by gas liquid chromatography, GLC (Bowyer et al. 1962, see bibliography) including some modifications to the original technique: 2h at 65°C instead of 1h at 80°C, three washes with hexane instead of two and no wash with water.

To get rid of traces of organic solvents, lipid fractions I and II are warmed to about 125°C for about 15 minutes under inert atmosphere.

Fat was analyzed according to the American Oil Chemist's Society (AOCS). The following criteria have been used to analyze the lipids extracted: saponification and Wijs iodine indexes and moisture-volatile matter levels. Cholesterol content has also been determined by the method of Plummer 1987 (see bibliography). The same analyzes and others have been made by an independent laboratory under Professor Robert Ackman's supervision (Canadian Institute of Fisheries Technology, DalTech, Dalhousie University, Halifax, Nova Scotia, Canada). This includes Wijs iodine index, peroxide and anisidine values, lipid class composition, fatty acid composition, free fatty acid FAME, cholesterol, tocopherol, all-*trans* retinol, cholecalciferol, asthaxanthin and canthaxantin contents.

Table 1 shows that higher levels of lipids are extracted from dry krill by acetone followed by ethanol as compared to the classical procedure of Folch et al. (1957).

Table 2 shows the results of lipid extraction from frozen *Euphausia pacifica*, a species of krill from Pacific Ocean. Assuming an eighty percent content of water, the lipid content is comparable to dry krill as shown in Table 1. Isopropanol, *t*-butanol and ethyl acetate, as solvent for the second extraction, give a yield less important than ethanol, but are not necessarily less effective in lipid recovery since ethanol carries more impurities than isopropanol, *t*-butanol or ethyl acetate. Then, they can be used as second solvent after acetone as well. Variations between results from acetone extractions are mainly due to the water-oil separations. These separations are influenced by the quantity of residual acetone in the water-oil solution after acetone evaporation. This quantity of acetone varies from an experiment to another, because the evaporation system used at a small scale is less reproducible (at the industrial scale, the evaporation step will be optimized). Single solvents have also been tested to extract the totality of lipids from krill. This shows that ethyl acetate (1,37% extraction rate), as hexane (0,23% extraction rate) are not good solvents, compared to acetone alone (1,86% extraction rate, and even greater extraction rates with an efficient acetone evaporation system).

One of the main advantages of the procedure is the removal of bacteria from extracts (lipid fraction and solid protein-rich material). Indeed, samples of *E. pacifica* incubated in different ratios of acetone at 4°C for 112 days have been inoculated on NA medium containing Bacto™ beef extract 0,3%, Bacto™ peptone 0,5% and Bacto™ agar 1,5% (Difco Laboratories, Detroit, USA) then incubated at room temperature or 4°C for 18 days. No significant bacterial growth was observed at a ratio of 1 volume of acetone per gram of krill. At higher proportions of acetone (2 volumes and 5 volumes), there was no bacterial growth at all, which means that acetone preserves krill samples. Acetone is known as an efficient bactericidal and viricidal agent (Goodman et al. 1980).

Table 3 shows the yield of lipids from *M. norvegica*. The percentage of lipids (3,67%) is comparable to the one obtained with *E. pacifica* (3,11%) shown in Table 2. Variations can be attributable to diet and time (season) of collection, which are different for those two species.

Table 4 shows the influence of grinding on the efficiency of extraction of *M. norvegica* lipids. These extractions were carried out under optimal conditions and show the definite advantage of the procedure over the classical method (4,46 % versus 3,30 %). It also shows that grinding may be an important factor when the species is large (4,46% versus 3,53 %).

Table 5 reports on lipid extraction from *Calanus*. Considerable quantities of lipids were obtained. Some variations in *Calanus* species composition may explain the variations between experiments 1 and 2 (8,22 % and 10,90 % of fresh weight).

Tables 6-8 report the total amount of lipids extracted from fish tissue. The method of the present invention was demonstrated on mackerel, trout and herring. The method was demonstrated on peripheral tissues (mainly muscles) and viscera. Advantageously, the present method would permit the recovery of valuable lipid fractions from parts of fish that are usually wasted after the withdrawal of fillets of the fish. Those fish tissues not used after the transformation of the fish for human consumption could be stored in acetone, and lipids extracted therefrom in accordance with the present invention even if the method Folch [1957] recovers more lipid than our method. Indeed small amounts of lipids from mackerel (0.52% from viscera and 1,45% from tissues) have been extracted by the method of Folch after a first extraction with acetone and ethanol as described in the present invention. Comparative extractions with the method described in the present invention carried out in parallel with the method of Folch on trout and herring show superior recovery with the latter. However, it is noteworthy that the Folch method can not be applied for the recovery of lipids for commercial uses (because of toxicity).

In Tables 9 to 11, are shown results of lipids extraction from shark liver tissues. There is no marked difference in results between techniques within a species. Table 12 shows the fatty acid composition of krill oil (*e. pacifica*) following extraction in various solvents.

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Tables 13 shows some characteristics features of fraction I (acetone) and fraction II (alcohol or ethyl acetate) for krill oil (*e. pacifica*). First, the saponification index of fraction I (130,6) indicates that this fraction contains fatty acids with longer chains, compared to fraction II (185,7). The Wijs iodine index of fraction I shows that this fraction contains high levels of polyunsaturated fatty acids. As compared to olive oil which has an index of 81.1. It explains why fraction I is liquid at room temperature. It is well known that unsaturated fatty acids have a fusion point inferior to the one of their saturated homologues. The same observations are made for fraction II which has a iodine index of 127,2. The fatty acid composition shown in Table 12 corroborates these iodine indexes: fraction I has a high percentage (30,24%) of polyunsaturated fatty acids (pentaenes+hexaenes) and so fraction II (22,98%). Finally, Table 13 shows also that fraction I is comprised of 10,0% of volatile matter and humidity after evaporation of the solvent. For the same test, the fraction II gives a value of 6,8%. To get rid of traces of solvents, it is important to briefly heat (to about 125°C, for about 15 min) the oil under nitrogen.

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Results on krill oils obtained in accordance with the method of the present invention (fraction I extracted with acetone and fraction II extracted with ethyl acetate) are provided in Tables 13, 14, 15, 16, 17 and 18. It is noteworthy to mention that in Table 18, the carotenoids content was significantly high as measured in terms of two carotenoids namely asthaxanthin and canthaxanthin. Indeed, duplicates analyzes revealed values of 92 to 124 µg/g of lipid fraction for asthaxanthin and 262 to 734 µg/g for canthaxanthin. Thus, for the purpose of the present invention it may be said that the krill extract comprises asthaxanthin at least 75 and preferably at least 90 µg/g of lipid fraction. In the case of canthaxanthin, at least 250 and preferably at least 270 µg/g of lipid fraction. Low values for peroxide and anisidine are advantageous and are due to the presence of high levels of natural antioxidants

(astaxanthin and canthaxanthin). These compounds are indicative of favourable pharmaceutical or cosmetological properties of the krill extract whereby high levels of carotenoids indicate excellent transdermal migration characteristics. Thus, krill extract is a good candidate for transdermal delivery of medicines.

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Table 19 shows the best mode of the method in accordance with the present invention for lipid extraction of aquatic animal tissues.

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Table 20 shows that the enzyme activity of the solid fraction is maintained following the method of the present invention. Indeed, the demonstration was completed for solid krill residue obtained after successive acetone and ethyl acetate extraction. Proteolytic activities were measured by the liberation of amino groups by spectrophotometric assay using o-phthalaldehyde as reagent. Protein concentrations were measured by the Bradford method. Soluble proteins were extracted with water and added to a 10% lactoserum protein concentrate obtained by ultrafiltration. At the end of incubation at 37°C in 50mM potassium phosphate buffer, trichloroacetic acid was added and the amount of NH<sub>3</sub> group was measured in the supernatant according to the method of Church et al. [1983, J Dairy Sci 66: 1219-1227].

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Figures 1 to 6 show chromatograms of fatty acid composition of *E. pacifica* lipids. On each of them, high proportions of 20:5 and 22:6 fatty acids (characteristic of marine and aquatic oils) are noticeable and represented by two distinct peaks. Data are shown in Table 12.

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Variations in lipid patterns of neutral lipids (from Figure 7 to Figure 11) from one species to another are attributable to the differences in food sources. Within a species (*E. pacifica*, for example) there is no marked variation between lipid patterns obtained from different techniques of lipid extraction. Concerning phospholipids (Figure 12 to Figure 16), the opposite is observed: variations are explained by the different extraction processes of lipids since the same species do not lead to the same lipid pattern. Lipids from shark species (extracted by the mentioned methods)

and commercial cod-liver oil (sample available from Uniprix drugstores, Province of Québec, Canada) are mainly composed of neutral lipids as opposed to phospholipids.

5 The influence of the volume of solvent and incubation time on the efficiency of the acetone to extract lipids from *E. pacifica* is illustrated in Figures 17 and 18, respectively. A ratio of 1:6 (w/v) produced optimal yield with near complete extraction after 2h. The second extraction step has been experimented with ethanol. The volume of this solvent does not appear to be critical since the same yield was  
10 obtained with different volumes of ethanol (Figure 19), but incubations time in ethanol should be at least 30 minutes as indicated by the results on Figure 20.

One of the inventors, Dr. Adrien Beaudoin, has ingested the different lipid fractions of krill. No side effect profile was observed.

15 Although the invention has been described above with respect with one specific form, it will be evident to a person skilled in the art that it may be modified and refined in various ways. It is therefore wished to have it understood that the present invention should not be limited in scope, except by the terms of the following claims.

20 Demonstration that krill residue, obtained after acetone and ethyl acetate extraction, contains enzyme proteolytic activities. Proteolytic activities were measured by the liberation of amino groups by spectrophotometric assay using o-phthaldialdehyde as reagent. Protein concentrations were measured by the Bradford method.

25 The enzyme source was the residue obtained after acetone and ethyl acetate extractions of lipids. Soluble proteins were extracted with water and added to a 10% lactoserum protein concentrate obtained by ultrafiltration.

30 At the end of incubation at 37°C in 50 mM potassium phosphate buffer, trichloroacetic acid was added and the amount of NH<sub>3</sub> groups were measured in the supernatant according to Church and al. 1983.

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**TABLE 1. EXTRACTION OF DRY KRILL LIPIDS (*E. pacifica*)**

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) $\pm$ s.d.
5	1- acetone <sup>a)</sup>	8,00		
	ethanol <sup>b)</sup>	7,60	<b>15,60</b>	
	2- "	19,70		
		6,90	<b>26,60</b>	
10	3- "	8,15		
		11,20	<b>19,35</b>	
	4- "	6,80		
15		13,60	<b>20,40</b>	
				<b>20,49<math>\pm</math>3,95</b>
	5- chlor : MeOH <sup>c)</sup>		<b>15,50</b>	
20	6- "		<b>14,90</b>	
				<b>15,20<math>\pm</math>0,30</b>

Determinations in triplicates (variation < 5 %).

<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), no incubation.

25 <sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 night at 4°C, following a first extraction with acetone.

<sup>c)</sup> :Folch et al. 1957.

**30 TABLE 2. EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)**

Exp. No.	Technique	Yield (%)	Total (%)	Mean (%) $\pm$ s.d.
35	1- acetone <sup>a)</sup>	1,17		
	ethanol <sup>b)</sup>	1,23	<b>2,40</b>	
	2- "	3,05		
		1,09	<b>4,14</b>	
40	3- "	1,53		
		1,26	<b>2,79</b>	
				<b>3,11<math>\pm</math>0,91</b>
	4- acetone <sup>a)</sup>	2,45		
45	isopropanol <sup>b)</sup>	0,70	<b>3,15</b>	
	5- "	1,80		
		0,80	<b>2,60</b>	
50	6- "	1,60		
		0,80	<b>2,40</b>	
				<b>2,72<math>\pm</math>0,39</b>

**TABLE 2 (continued). EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)**

	<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>	<u>Mean (%) <math>\pm</math> s.d.</u>
5	7-	acetone <sup>a)</sup> <i>t</i> -butanol <sup>c)</sup>	2,15 0,47	<b>2,62</b>	
10	8-	"	2,11 0,40	<b>2,51</b>	
	9-	"	2,37 0,45	<b>2,82</b>	
15	10-	acetone <sup>a)</sup> ethyl acetate <sup>b)</sup>	2,28 0,21	<b>2,49</b>	<b>2,65<math>\pm</math>0,16</b>
20	11-	"	1,09 0,16	<b>1,25</b>	
	12-	"	2,54 0,09	<b>2,63</b>	
25	13-	combined acetone-ethanol <sup>d)</sup>		<b>3,28</b>	<b>2,12<math>\pm</math>0,76</b>
	14-	"		<b>3,02</b>	
30	15-	"		<b>3,25</b>	
	16-	ethyl acetate <sup>e)</sup>		<b>1,32</b>	<b>3,18<math>\pm</math>0,14</b>
35	17-	"		<b>1,49</b>	
	18-	"		<b>1,31</b>	<b>1,37<math>\pm</math>0,10</b>
40	19-	hexane <sup>e)</sup>		<b>0,31</b>	
	20-	"		<b>0,18</b>	
	21-	"		<b>0,20</b>	
45	22-	chlor:MeOH <sup>f)</sup>		<b>2,37</b>	<b>0,23<math>\pm</math>0,07</b>

**TABLE 2 (continued). EXTRACTION OF FROZEN KRILL LIPIDS (*E. pacifica*)**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>	<u>Mean (%) ± s.d.</u>
5	23-	"	2,07	
	24-	"	2,62	
				2,35±0,28
10	Determinations in triplicates (variation < 5 %).			
	a) :Extraction made with a sample-solvent ratio of 1:6 (w/v), incubated 2 h at 4°C.			
	b) :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.			
	c) :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 25°C, following a first extraction with acetone.			
15	d) :Extraction made with a sample-acetone-ethanol ratio of 1:5:5 (w/v/v), incubated 2 h at 4°C.			
	e) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2 h at 4°C.			
	f) : Folch et al. 1957.			

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**TABLE 3. EXTRACTION OF FROZEN KRILL LIPIDS(*M. norvegica*)**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>	<u>Mean (%) ± s.d.</u>
25	1-	acetone <sup>a)</sup>	1,82	
		ethanol <sup>b)</sup>	1,82	3,64
	2-	"	1,15	
			2,35	3,50
30	3-	"	1,68	
			2,19	3,87
				3,67±0,15
35	Determinations in triplicates (variation < 5 %).			
	a) :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.			
	b) :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.			

**TABLE 4. INFLUENCE OF GRINDING ON EXTRACTION OF FROZEN KRILL LIPIDS (*M. norvegica*)**

	<u>Exp. No.</u>	<u>Technique</u>	<u>Krill ground before 1<sup>st</sup> extraction</u>	<u>Yield (%)</u>	<u>Total (%)</u>
5	1-	acetone <sup>a)</sup> ethanol <sup>b)</sup>	yes	3,10 1,07	4,17
10	2-	"	no	2,14 1,39	3,53
	3-	"	yes	3,32 1,14	4,46
15	4-	chlor : MeOH <sup>c)</sup>	yes		3,30
	5-	"	yes		3,26

Determinations in triplicates (variation < 5 %).

<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:6, incubated 2 h at 4°C.

<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:2, incubated 30 min at 4°C, following a first extraction with acetone.

<sup>c)</sup> :Folch et al. 1957.

**TABLE 5. EXTRACTION OF FROZEN *Calanus* LIPIDS (*Calanus* sp.)**

	<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>	<u>Mean (%) ± s.d.</u>
30	1-	acetone <sup>a)</sup> ethanol <sup>b)</sup>	6,18 2,04	<b>8,22</b>	
	2-	"	8,64 2,26	<b>10,90</b>	
35					<b>9,56±1,34</b>

Determinations in triplicates (variation < 5 %).

<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.

<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.

**TABLE 6. EXTRACTION OF FRESH FISH LIPIDS (Mack rel)**

	<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>
5	1- viscera fish 1	acetone <sup>a)</sup> ethanol <sup>b)</sup>	6,11 0,59	<b>6,70</b>
	2- tissues fish 1	"	3,78 0,91	<b>4,69</b>
10	3- viscera fish 2	"	10,46 0,57	<b>11,03</b>
15	4- issues fish 2	"	6,65 1,41	<b>8,06</b>
	5- viscera fish 3	"	8,39 0,66	<b>9,05</b>
20	6- tissues fish 3	"	5,27 0,97	<b>6,24</b>
	7- viscera fish 4	"	8,47 0,69	<b>9,16</b>
25	8- tissues fish 4	"	8,40 1,02	<b>9,42</b>
30	9- viscera fish 1	chlor:MeOH <sup>c)</sup>		<b>0,52</b>
	10- tissues fish 1	"		<b>1,45</b>

35 <sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubation time:

- fish 1 viscera: 4h, fish 1 tissues: 23h
- fish 2 viscera: 23h45, fish 2 tissues: 45h30
- fish 3 viscera: 8 days 2h20, fish 3 tissues: 8 days 22h30
- fish 4 viscera: 17 days 23h, fish 4 tissues: 18 days 2h25.

40 <sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1h at 4°C, following a first extraction with acetone.

<sup>c)</sup> :Folch et al. 1957, following extractions with acetone, then ethanol.

**TABLE 7. EXTRACTION OF FRESH FISH LIPIDS (Trout)**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>
5	1- viscera	acetone <sup>a)</sup>	34,70
		ethanol <sup>b)</sup>	2,18
			<b>36,88</b>
10	2- tissues	"	5,53
			1,17
			<b>6,70</b>
15	3- viscera	chlor:MeOH <sup>c)</sup>	39,81
	4- tissues	"	14,93
Determination in triplicates (variation < 5 %).			
<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°C.			
<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.			
<sup>c)</sup> :Folch et al. 1957.			

**TABLE 8. EXTRACTION OF FRESH FISH LIPIDS (Herring)**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>
25	1-tissues and viscera	acetone <sup>a)</sup>	2,09
		ethanol <sup>b)</sup>	0,68
			<b>2,77</b>
30	2-tissues and viscera	chlor:MeOH <sup>c)</sup>	5,95
Determination in triplicates (variation < 5 %).			
<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 1 night at 4°.			
<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:4 (w/v), incubated 1 h at 4°C, following a first extraction with acetone.			
<sup>c)</sup> :Folch et al. 1957.			

**TABLE 9. EXTRACTION OF FRESH SHARK LIVER LIPIDS (M. schmitti)**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>
40	1-	acetone <sup>a)</sup>	36,39
		ethyl acetate <sup>b)</sup>	4,48
			<b>40,87</b>
45	2-	ethyl acetate <sup>c)</sup>	36,68
	3-	chlor : MeOH <sup>d)</sup>	41,86
Determination in triplicates (variations <5 %).			
<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.			
<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.			
<sup>c)</sup> :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.			
<sup>d)</sup> :Folch et al. 1957.			

**TABLE 10. EXTRACTION OF FRESH SHARK LIVER LIPIDS (*G. galeus*).**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>
5	1- acetone <sup>a)</sup>	21,39	<b>26,66</b>
	ethyl acetate <sup>b)</sup>	5,27	
	2- ethyl acetate <sup>c)</sup>		<b>25,89</b>
10	3- chlor : MeOH <sup>d)</sup>		<b>29,99</b>

Determinations in triplicates (variations <5 %).

<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.

<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

<sup>c)</sup> :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.

<sup>d)</sup> :Folch et al. 1957.

**TABLE 11. EXTRACTION OF FRESH SHARK LIVER LIPIDS (Angel Shark)**

<u>Exp. No.</u>	<u>Technique</u>	<u>Yield (%)</u>	<u>Total (%)</u>
25	1- acetone <sup>a)</sup>	19,23	<b>28,21</b>
	ethyl acetate <sup>b)</sup>	8,98	
	2- ethyl acetate <sup>c)</sup>		<b>39,22</b>
30	3- chlor : MeOH <sup>d)</sup>		<b>39,23</b>

Determinations in triplicates (variations <5 %).

<sup>a)</sup> :Extraction made with a sample-solvent ratio of 1:9 (w/v), incubated 2h at 4°C.

<sup>b)</sup> :Extraction made with a sample-solvent ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

<sup>c)</sup> :Extraction made with a sample-solvent ratio of 1 :9 (w/v), incubated 2h at 4°C.

<sup>d)</sup> :Folch et al. 1957.

**TABLE 12. FATTY ACID COMPOSITION (*E. pacifica*)**

<u>Solvent</u>	<u>Saturated</u>	<u>Unsaturated Mono</u>	<u>Di</u>	<u>Poly</u>	<u>H-Poly</u>	<u>Unidentified</u>
chlo-meth	26.18	22.54	1.91	3.23	26.34	19.8
45 acetone	21.4	22.18	1.75	3.7	24.52	26.46
acetone	19.09	22.11	2.03	3.48	30.24	23.03
ethanol	28.07	22.92	2.14	3.07	27.78	16.03
50 t-butanol	32.63	24.96	1.86	2.86	17.86	19.83
ethyl acetate	22.68	25.77	2.17	2.88	22.98	23.51

Data expressed in percentage of total fatty acids (%).

**TABLE 13. CHARACTERISTICS OF KRILL OIL (*E. pacifica*)**

			independent laboratory <sup>a)</sup>	handbook <sup>b)</sup>
5	<u>Saponification index</u>			
	Fraction I <sup>c)</sup>	130,6	---	---
	Fraction II <sup>d)</sup>	185,7	---	---
	Olive oil	192,0 <sup>e)</sup>	---	189,7
10	<u>Wijs iodine index</u>			
	Fraction I <sup>c)</sup>	185,2	172,5	---
	Fraction II <sup>d)</sup>	127,2	139,2	---
15	Olive oil	85,3 <sup>e)</sup>	---	81,1
	<u>Cholesterol content (%)</u>			
	Fraction I <sup>c)</sup>	2,1	1,9	---
20	Fraction II <sup>d)</sup>	3,7	3,0	---
	Olive oil	0,2 <sup>e)</sup>	---	---
	<u>Volatile matter and moisture levels (%)</u>			
25	Fraction I <sup>c)</sup>	10,0	---	---
	Fraction II <sup>d)</sup>	6,8	---	---
	<u>Peroxide value (meq peroxide/kg oil)</u>			
30	Fraction I <sup>c)</sup>	---	0,0	---
	Fraction II <sup>d)</sup>	---	0,0	---
	<u>p-Anisidine value (g<sup>-1</sup> absorption)</u>			
35	Fraction I <sup>c)</sup>	---	0,1	---
	Fraction II <sup>d)</sup>	---	5,5	---

<sup>a)</sup>: Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

40 <sup>b)</sup>: Harwood and Geyer 1964.

<sup>c)</sup>: Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

<sup>d)</sup>: Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

<sup>e)</sup>: Extra virgin olive oil cold compressed from Bertolli <sup>TM</sup>.

45



**TABLE 14. LIPID CLASS COMPOSITION OF KRILL OIL (AREA %) (*E. pacifica*)**Triglycerides

5	Fraction I <sup>a)</sup>	19,0±0,7
	Fraction II <sup>b)</sup>	66,5± 2,3

Hydrocarbons

10	Fraction I <sup>a)</sup>	trace
	Fraction II <sup>b)</sup>	1,3± 0,1

Free fatty acids

15	Fraction I <sup>a)</sup>	23,7± 1,1
	Fraction II <sup>b)</sup>	20,3± 0,3

Monoglycerides

20	Fraction I <sup>a)</sup>	1,4± 0,3
	Fraction II <sup>b)</sup>	0,5± 0,1

Phospholipids or other polar material

25	Fraction I <sup>a)</sup>	54,1± 6,1
	Fraction II <sup>b)</sup>	8,5 ±1,6

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

30 <sup>a)</sup> : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

<sup>b)</sup> : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

**TABLE 15. FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%) (*E. pacifica*)**

	<u>Fatty acids</u>	<u>Fraction I <sup>a)</sup></u>	<u>Fraction II <sup>b)</sup></u>
	12 :0	0,0	0,1
40	13 :0	0,2	0,1
	ISO 14 :0	0,4	0,6
	14:0	4,2	7,6
	ISO 15:0	0,5	0,7
	ANT 15:0	0,2	0,2
45	15:0	0,6	1,0
	ISO 16:0	0,2	0,3
	ANT 16:0	0,2	0,2
	16:0	14,1	21,6
	7MH	0,6	0,9
50	ANT 17:0	0,1	0,3
	17:0	2,8	3,7
	18:0	1,0	1,6
	20:0	0,1	0,3
55	Saturates	25,2	39,2

**TABLE 15 (continued). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%)**  
**(*E. pacifica*)**

	<u>Fatty acids</u>	<u>Fraction I <sup>a)</sup></u>	<u>Fraction II <sup>b)</sup></u>
5	14:1	0,4	0,5
	15:1	0,1	0,2
	16:1 n-7	6,6	7,8
	16:1 n-5	0,6	0,2
10	17:1	0,6	0,7
	18:1 n-9	8,0	9,8
	18:1 n-7	4,2	5,6
	18:1 n-5	0,1	0,1
	20:1 n-9	0,3	0,4
15	20:1 n-7	0,3	0,4
	20:1 n-5	0,3	0,4
	22:1 n-11 +13	0,1	0,2
	Monoenes	21,6	26,3
20	16:2 n-6	0,6	1,2
	16:2 n-4	1,3	1,3
	18:2 n-7	0,1	0,2
	18:2 n-6	2,0	1,8
	18:2 n-4	0,1	0,1
25	20:2 NMID	0,2	0,2
	20:2 n-6	0,1	0,1
	Dienes	4,4	4,9
30	16:3 n-4	1,4	1,2
	18:3 n-6	0,4	0,3
	18:3 n-4	0,2	0,2
	18:3 n-3	3,2	3,0
	18:3 n-1	0,1	0,1
35	20:3 n-3	0,1	0,1
	Trienes	5,4	4,9
	16:4 n-3	0,9	0,7
	16:4 n-1	1,0	0,8
40	18:4 n-3	9,2	7,4
	18:4 n-1	0,1	0,0
	20:4 n-6	0,7	0,5
	20:4 n-3	0,7	0,3
	Tetraenes	12,6	9,7
45	20:5 n-3	17,4	8,6
	21:5 n-3	0,7	0,5
	22:5 n-6	0,2	0,1
	22:5 n-3	0,5	0,3
50	Pentaenes	18,8	9,5

**TABLE 15 (continued). FATTY ACID COMPOSITION OF KRILL OIL (WT/WT%)  
(*E. pacifica*)**

	<u>Fatty acids</u>	<u>Fraction I <sup>a)</sup></u>	<u>Fraction II <sup>b)</sup></u>
5	22:6 n-3 Hexaenes	13,2	6,6
10	Iodine value calculated	214,8	145,1

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

<sup>a)</sup> : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

15 <sup>b)</sup> : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

**TABLE 16. KRILL LIPID FREE FATTY ACID FAME (WT/WT%) (*E. pacifica*)**

	<u>Fatty acids</u>	<u>Fraction I <sup>a)</sup></u>	<u>Fraction II <sup>b)</sup></u>
20	12:0	0,5	0,1
	13:0	0,2	0,0
25	ISO 14:0	0,2	0,2
	14:0	1,3	2,6
	ISO 15:0	0,3	0,3
	ANT 15:0	0,1	0,1
	15:0	0,2	0,5
30	ISO 16:0	0,1	0,2
	ANT 16:0	0,2	0,1
	16:0	3,3	10,6
	7MH	0,6	0,8
	ANT 17:0	0,2	0,2
35	Phytanic	0,2	0,0
	17:0	0,5	0,8
	18:0	0,2	0,6
	20:0	0,3	0,2
	22:0	0,0	0,1
40	Saturates	8,4	17,4
	14:1	0,2	0,2
	15:1	0,2	0,1
	16:1 n-9	0,5	0,0
45	16:1 n-7	5,2	6,8
	16:1 n-5+17:0	0,1	0,1
	17:1	0,6	0,7
	18:1 n-9	7,0	11,4
	18:1 n-7	4,9	9,3
50	18:1 n-5	0,1	0,3
	20:1 n-11	0,2	0,3
	20:1 n-9	0,1	0,3
	22:1 n-11+13	0,1	0,2
	24:1 n-9	0,0	0,1
55	Monoenes	19,2	29,8

**TABLE 16 (continued). KRILL LIPID FREE FATTY ACID FAME (WT/WT%) (*E. pacifica*)**

	<u>Fatty acids</u>	<u>Fraction I <sup>a)</sup></u>	<u>Fraction II <sup>b)</sup></u>
5	16:2 n-6	0,4	0,9
	16:2 n-4	1,2	1,0
	18:2 n-7	0,1	0,2
	18:2 n-6	2,4	2,6
	18:2 n-4	0,1	0,1
10	20:2 n-6	0,1	0,1
	Dienes	4,3	4,9
	16:3 n-4+17:1	1,4	0,9
15	16:3 n-3+18:0	0,2	0,5
	18:3 n-6	0,4	0,3
	18:3 n-4	0,1	0,1
	18:3 n-3	3,3	3,4
	18:3 n-1	0,1	0,1
20	20:3 n-6	0,1	0,1
	20:3 n-3	0,1	0,2
	Trienes	5,7	5,6
25	16:4 n-3	0,6	0,3
	16:4 n-1	1,0	0,6
	18:4 n-3	9,8	6,2
	18:4 n-1	0,1	0,1
	20:4 n-6	1,7	1,4
30	20:4 n-3	0,6	0,5
	22:4 n-3	0,3	0,3
	Tetraenes	14,1	9,4
	18:5 n-3	0,2	0,1
35	20:5 n-3	26,4	17,4
	21:5 n-3	0,9	0,6
	22:5 n-6	0,0	0,1
	22:5 n-3	0,7	0,5
	Pentaenes	28,2	18,7
40	22:6 n-3	20,5	14,4
	Hexaenes	20,5	14,4
45	Iodine value calculated	291,6	220,3

Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.

<sup>a)</sup> : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.

<sup>b)</sup> : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.

**TABLE 17. TOCOPHEROL, ALL-*trans* RETINOL AND CHOLECALCIFEROL CONTENT IN KRILL OIL (*E. pacifica*)**

<u>alpha-tocopherol by HPLC (IU)</u>			
5	Fraction I <sup>a)</sup>	0,91	
	Fraction II <sup>b)</sup>	0,83	
<u>gamma-tocopherol by HPLC µg/g</u>			
10	Fraction I <sup>a)</sup>	Tr	
	Fraction II <sup>b)</sup>	Tr	
<u>delta-tocopherol by HPLC µg/g</u>			
15	Fraction I <sup>a)</sup>	N.D.	
	Fraction II <sup>b)</sup>	N.D.	
<u>all-<i>trans</i> retinol by HPLC (IU)</u>			
	Fraction I <sup>a)</sup>	395,57	
	Fraction II <sup>b)</sup>	440,47	
20	<u>cholecalciferol by HPLC (IU)</u>		
	Fraction I <sup>a)</sup>	N.D.	
	Fraction II <sup>b)</sup>	N.D.	
25	Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.		
	Data expressed per gram of krill oil.		
	<sup>a)</sup> : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.		
	<sup>b)</sup> : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.		
30	TR = trace		
	N.D. = not detected		
	Conversion : Vitamin	alpha-tocopherol	mg/g oil x 1,36 = International Unit
		All- <i>trans</i> retinol	µg/g ÷ 0,3 = International Unit

**TABLE 18. ASTAXANTHIN AND CANTHAXANTHIN CONTENT OF KRILL OIL (*E. pacifica*)**

<u>Astaxantin (µg/g oil)</u>			
40	Fraction I <sup>a)</sup>	93,1	
	Fraction II <sup>b)</sup>	121,7	
<u>Canthaxanthin (µg/g oil)</u>			
45	Fraction I <sup>a)</sup>	270,4	
	Fraction II <sup>b)</sup>	733,0	
50	Data from Professor Robert Ackman's laboratory, Canadian Institute of Fisheries Technology, Halifax, Nova Scotia.		
	<sup>a)</sup> : Extraction made with a sample-acetone ratio of 1:6 (w/v), incubated 2h at 4°C.		
	<sup>b)</sup> : Extraction made with a sample-ethyl acetate ratio of 1:2 (w/v), incubated 30 min at 4°C, following a first extraction with acetone.		

**TABLE 19. OPTIMAL CONDITIONS FOR LIPID EXTRACTION OF AQUATIC ANIMAL TISSUES (suggested procedure)**

	<u>STEP</u>	<u>CONDITIONS</u>
5	Grinding (if particles > 5mm)	4°C
	Lipid extraction	sample-acetone ratio of 1:6 (w/v) 2h (including swirling 20 min)
10		4°C
	Filtration	organic solvent resistant filter under reduced pressure
15	Washing	sample-acetone ratio of 1:2 (w/v) pure and cold acetone
	Filtration	organic solvent resistant filter under reduced pressure
20	Evaporation	under reduced pressure
	Oil-water separation	4°C
25	Lipid extraction	<u>sample: ethyl acetate</u> ratio of 1:2 (w/v) <sup>a)</sup> pure <u>ethyl acetate</u> 30 min 4°C <sup>b)</sup>
30	Filtration	organic solvent resistant filter under reduced pressure
	Evaporation	under reduced pressure
35	<sup>a)</sup> : Ethanol can be replaced by isopropanol, <i>t</i> -butanol or ethyl acetate. <sup>b)</sup> : 25 °C when using <i>t</i> -butanol.	

**TABLE 20: PROTEOLYTIC ACTIVITY OF KRILL RESIDU USING LACTOSERUM AS THE SUBSTRATE, AT 37 °C, PH 7.0 FOR A RATIO ENZYME:SUBSTRATE OF 1:43**

	<u>Time</u> <u>(min)</u>	<u>Amino acids released</u> <u>(μmoles)</u>	<u>Enzymatic rate</u> <u>(μmoles/min)</u>	<u>Specific enzymatic</u> <u>activity</u> <u>(μmoles/min/mg*)</u>
45	15	28.76	1.917	0.164
	30	43.74	0.999	0.125
	170	98.51	0.322	0.050
	255	177.26	0.308	0.060
50				

\* total quantity of enzymes in hydrolysis media

We claim:

1. A method for extracting lipid fractions from marine and aquatic animal material, said method comprising the steps of:
  - (a) placing marine and aquatic animal material in a ketone solvent, preferably acetone to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;
  - (b) separating the liquid and solid contents;
  - (c) recovering a first lipid rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;
  - (d) placing said solid contents in an organic solvent selected from the group of solvents consisting of alcohol, preferably ethanol, isopropanol or *t*-butanol and esters of acetic acid, preferably ethyl acetate to achieve extraction of the remaining soluble lipid fraction from said marine and aquatic animal material;
  - (e) separating the liquid and solid contents;
  - (f) recovering a second lipid rich fraction by evaporation of the solvent from the liquid contents;
  - (g) recovering the solid contents.
2. A method as in claim 1 wherein during step (a), the solvent and animal material are homogenized.
3. A method as in claim 1 wherein during step (d), the solvent and solid contents are homogenized.
4. A method as in any of claims 1 to 3 wherein steps (b) and (d) are conducted under inert gas atmosphere.
5. A method as in any of claims 1 to 4 wherein steps (b) and (e) are effected by techniques selected from filtration, centrifugation and sedimentation.

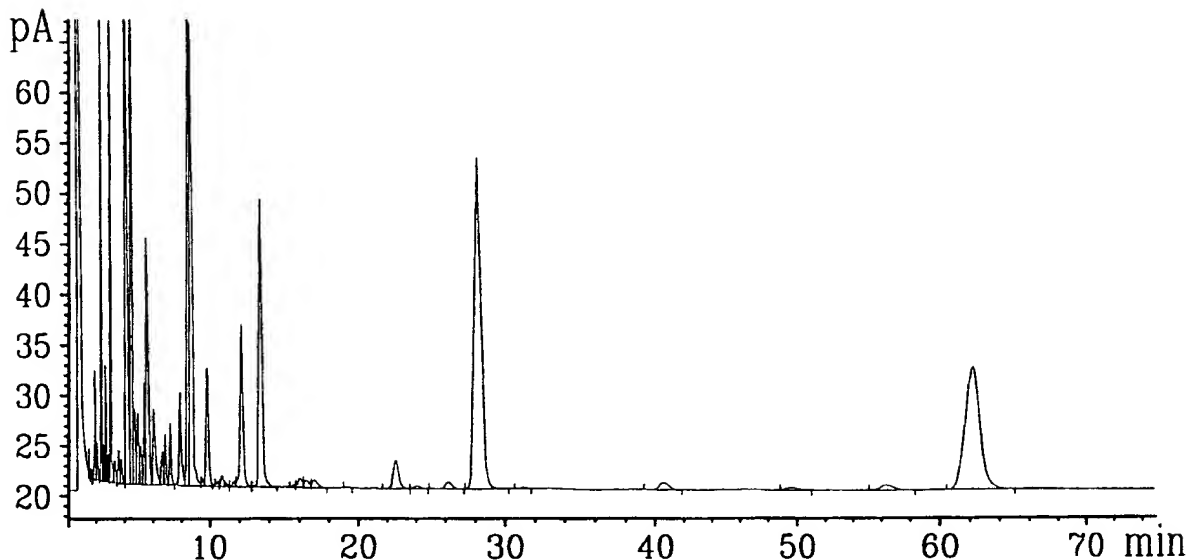
6. A method as in any of claims 1 to 5 wherein steps (c) and (f) are effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.
- 5 7. A method as in any of claims 1 to 6 wherein after step (b) and before step (c), the method additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).
- 10 8. A method as in any of claims 1 to 7 wherein after step (e) and before step (f), the method additionally comprises the intervening step of washing the solid contents with the organic solvent selected in step (d).
- 15 9. A method as in any of claims 1 to 8 wherein prior to step (a) the marine and aquatic animal material is finely divided, preferably to an average particle size of 5mm or less.
- 20 10. A method as in claims 1 to 9 wherein steps (a) and (b) are conducted at solvent temperatures of about 5°C or less.
- 25 11. A method as in claims 1 to 10 wherein said marine and aquatic animal is zooplankton.
12. A method as in claim 11 wherein said zooplankton is krill.
13. A method as in claim 12 wherein said zooplankton is *Calanus*.
14. A method as in claims 1 to 10 wherein said marine and aquatic animal is fish filleting by-products.



15. A method for extracting lipid fractions from marine and aquatic animal material selected from zooplankton and fish filleting by-products, preferably viscera, said method comprising the steps of:
- (a) placing said animal material in a ketone solvent, preferably acetone to achieve extraction of the soluble lipid fraction from said marine and aquatic animal material;
  - (b) separating the liquid and solid contents;
  - (c) recovering a lipid rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;
  - (d) recovering the solid contents.
16. A method as in claim 15 wherein the animal material is krill.
17. A method as in claim 15 wherein the animal material is *Calanus*.
18. A method as in claims 15 to 17 wherein during step (a), the solvent and animal material are homogenized.
19. A method as in any of claims 15 to 18 wherein steps (b) and (d) are conducted under inert gas atmosphere.
20. A method as in any of claims 15 to 19 wherein step (b) is effected by techniques selected from filtration, centrifugation and sedimentation.
21. A method as in any of claims 15 to 20 wherein step (c) is effected by techniques selected from vacuum evaporation, flash evaporation and spray drying.
22. A method as in any of claims 15 to 21 wherein after step (b) and before step (c), the method additionally comprises the intervening step of washing the solid contents with the solvent and adding the resulting washing solution to the liquid contents of step (b).

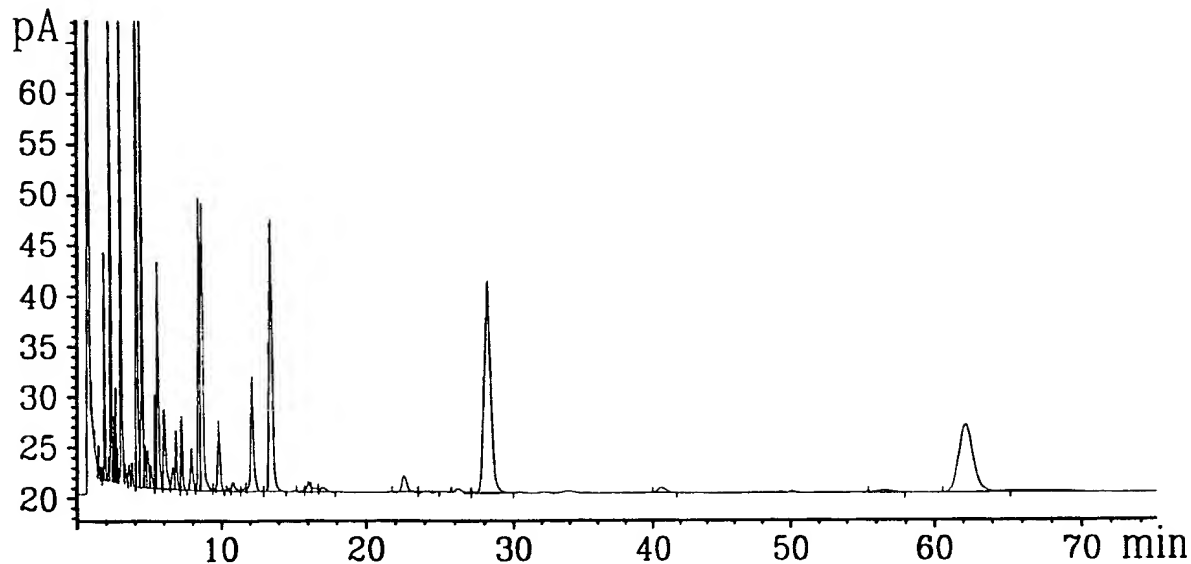
23. A method as in any of claims 15 to 22 wherein prior to step (a) the marine and aquatic animal material is finely divided, preferably to an average particle size of 5mm or less.
- 5 24. A method as in claims 15 to 23 wherein steps (a) and (b) are conducted at solvent temperatures of about 5°C or less.
25. A krill lipid extract characterized in that the carotenoid content in asthaxanthin is at least about 75 and preferably at least about 90: g/g of krill extract.
- 10 26. A krill lipid extract characterized in that the carotenoid content in canthaxanthin is at least about 250 µg/g and preferably at least about 270 µg/g of krill extract.
- 15 27. A method of lipid extraction as in claims 1 or 15 wherein the solid contents recovered in the last step consists of a dehydrated residue containing active enzymes.

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FIG. 1

1.263	4.521 - 16:1	11.637
1.455 - 12:0	4.684	12.145 - 18:3
1.625	4.891 - 16:1tr	13.458
1.812	5.121	15.626 - 20:0
1.876	5.426	16.045
2.056	5.570	16.482 - 20:1
2.173	6.037	17.017 - 20:1(cis11)
2.331 - 14:0	6.662	19.344 - 20:2
2.505	6.871	22.606 - 20:4(6,10,14,18)
2.591 - 14:1	7.235	24.103
2.682	7.925 - 18:0	26.247
2.802	8.439 - 18:1	28.287
2.855	8.640 - 18:1tr	31.295
3.078 - std 15:0	9.544	40.655
3.309	9.801 - 18:2	49.721
3.586	10.491	56.373
3.810	10.825	62.225
4.176 - 16:0	11.042	

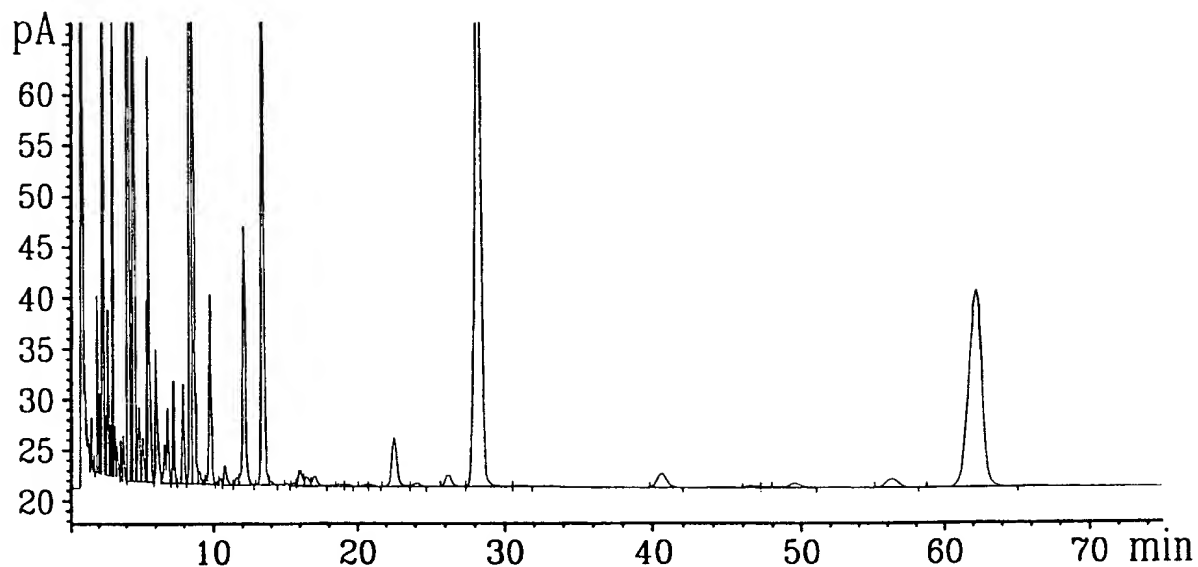
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FIS-2

1.217	3.806	9.786 - 18:2
1.264	4.157 - 16:0	10.484
1.454 - 12:0	4.515 - 16:1	10.813
1.624	4.680	11.590
1.812	4.891 - 16:1tr	12.136 - 18:3
1.876	5.028	13.447
2.055	5.109	15.623 - 20:0
2.171	5.421	16.025
2.330 - 14:0	5.562	16.466 - 20:1
2.505	6.031	17.021 - 20:1 (cis11)
2.591 - 14:1	6.642	22.585 - 20:4 (6,10,14,18)
2.680	6.870	24.100
2.800	7.230	26.217
2.854	7.910 - 18:0	28.241
3.077 - std 15:0	8.419 - 18:1	40.622
3.306	8.622 - 18:1tr	56.417
3.585	9.529	62.086

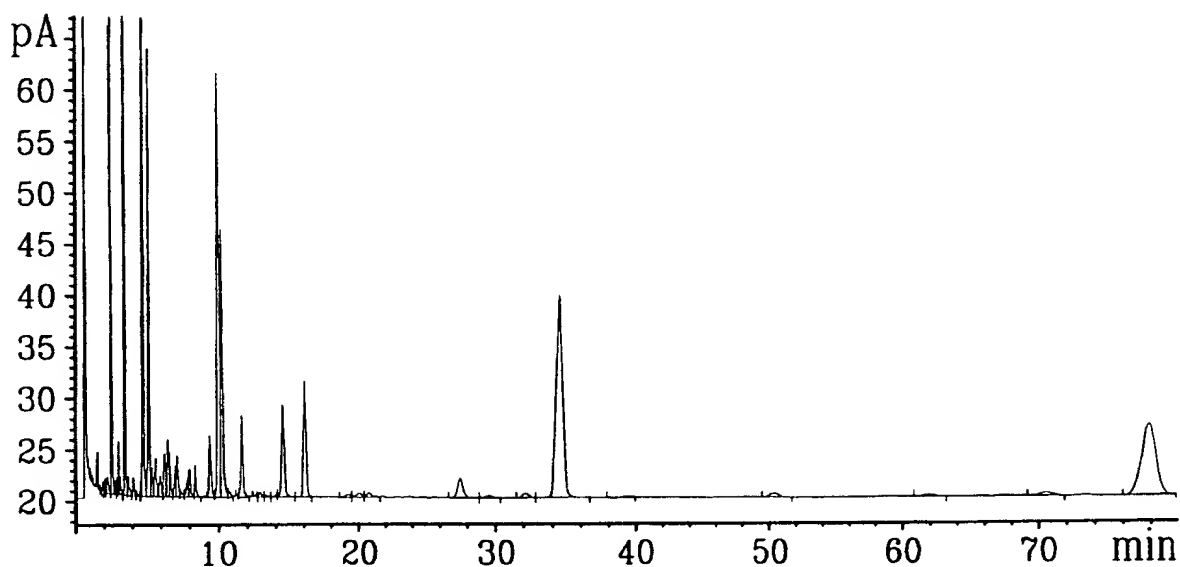
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FILE 3

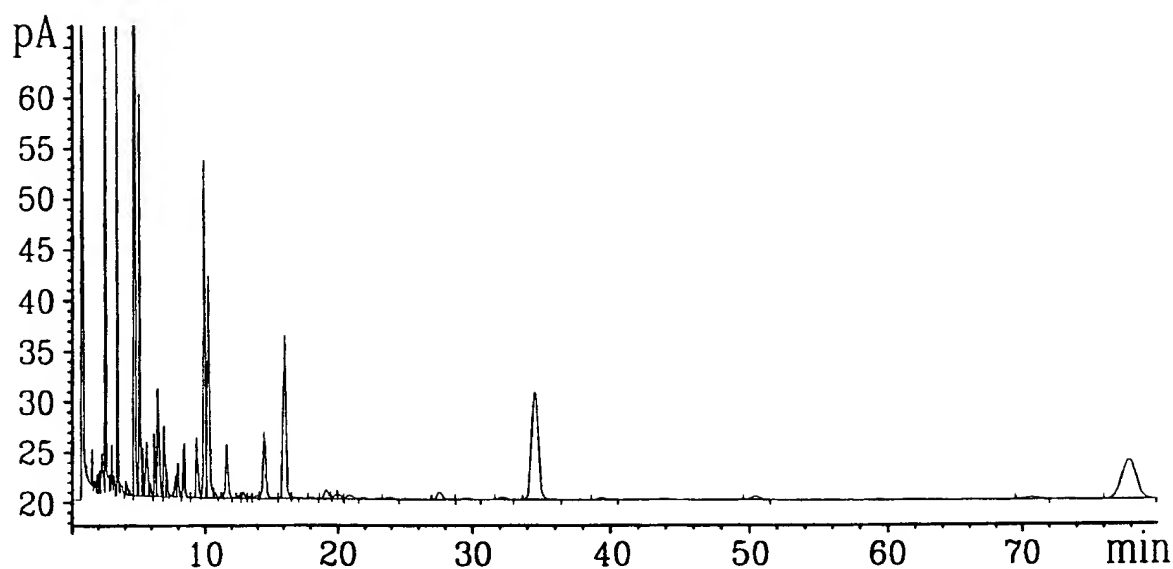
1.216	4.520 - 16:1	13.457
1.262	4.683	13.943
1.454 - 12:0	4.884 - 16:1tr	15.053
1.624	5.030	15.572 - 20:0
1.811	5.111	16.016
1.875	5.420	16.486 - 20:1
2.016	5.561	16.999 - 20:1(cis11)
2.054	6.031	18.762
2.174	6.642	19.303 - 20:2
2.330 - 14:0	6.868	20.474
2.505	7.226	21.027 - 20:3
2.589 - 14:1	7.908 - 18:0	22.575 - 20:4 (6,10,14,18)
2.679	8.444 - 18:1	24.071
2.799	8.639 - 18:1tr	26.215
2.854	9.005	28.333
2.981	9.536	31.180
3.074 - std 15:0	9.788 - 18:2	40.560
3.304	10.267	46.595
3.580	10.481	49.513
3.804	10.807	56.292
4.169 - 16:0	11.626	62.250
4.296	12.140 - 18:3	

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FIG. 4

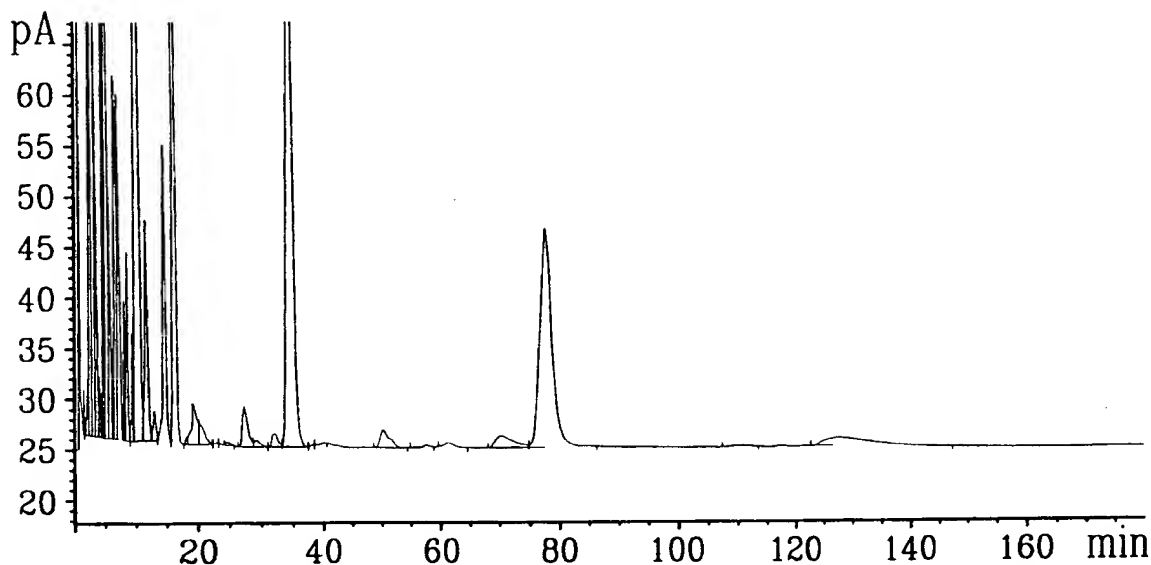
1.552 - 12:0	5.675 - 16:1tr	12.888
1.749	5.964	13.388
1.968	6.284	14.017
2.095	6.533	14.524 - 18:3
2.262	6.655	16.107
2.485	7.009	19.275 - 20:0
2.582 - 14:0	7.159	20.112 - 20:1 (cis11)
2.784	7.440	20.781 - 20:1
2.886 - 14:1	7.874	27.553
3.004	8.019	29.529
3.145	8.462	32.161
3.478 - std 15:0	9.411 - 18:0	34.614
3.720	10.000 - 18:1	39.240
4.088	10.249 - 18:1tr	50.374
4.325	10.716	61.892
4.793 - 16:0	11.357	70.568
5.196 - 16:1	11.647 - 18:2	77.894
5.406	12.519	

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1.556 - 12:0	5.977	14.540 - 18:3
1.753	6.294	16.133
1.972	6.546	16.805
2.104	7.020	18.131
2.304	7.166	19.154 - 20:0
2.590 - 14:0	7.889	19.875
2.892 - 14:1	8.030	20.099 - 20:1(cis11)
3.012	8.473	20.820 - 20:1
3.153	9.425 - 18:0	23.903
3.485 - std 15:0	10.010 - 18:1	27.583
3.710	10.260 - 18:1tr	29.570
4.096	10.735	32.195
4.203	11.394	34.597
4.333	11.661 - 18:2	39.334
4.800 - 16:0	12.540	50.452
5.206 - 16:1	12.909	70.660
5.417	13.402	77.895 - 24:0
5.711 - 16:1tr	14.010	

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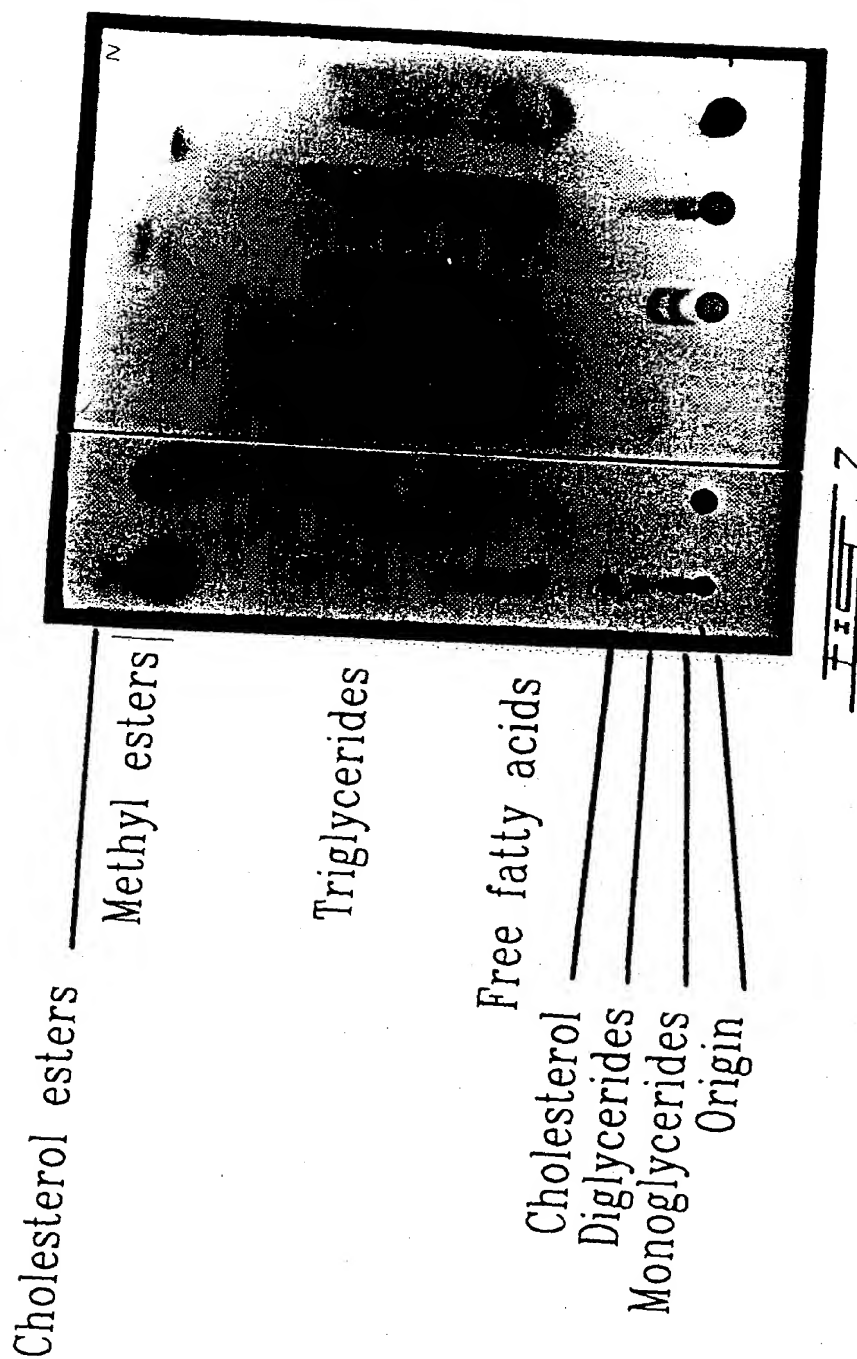


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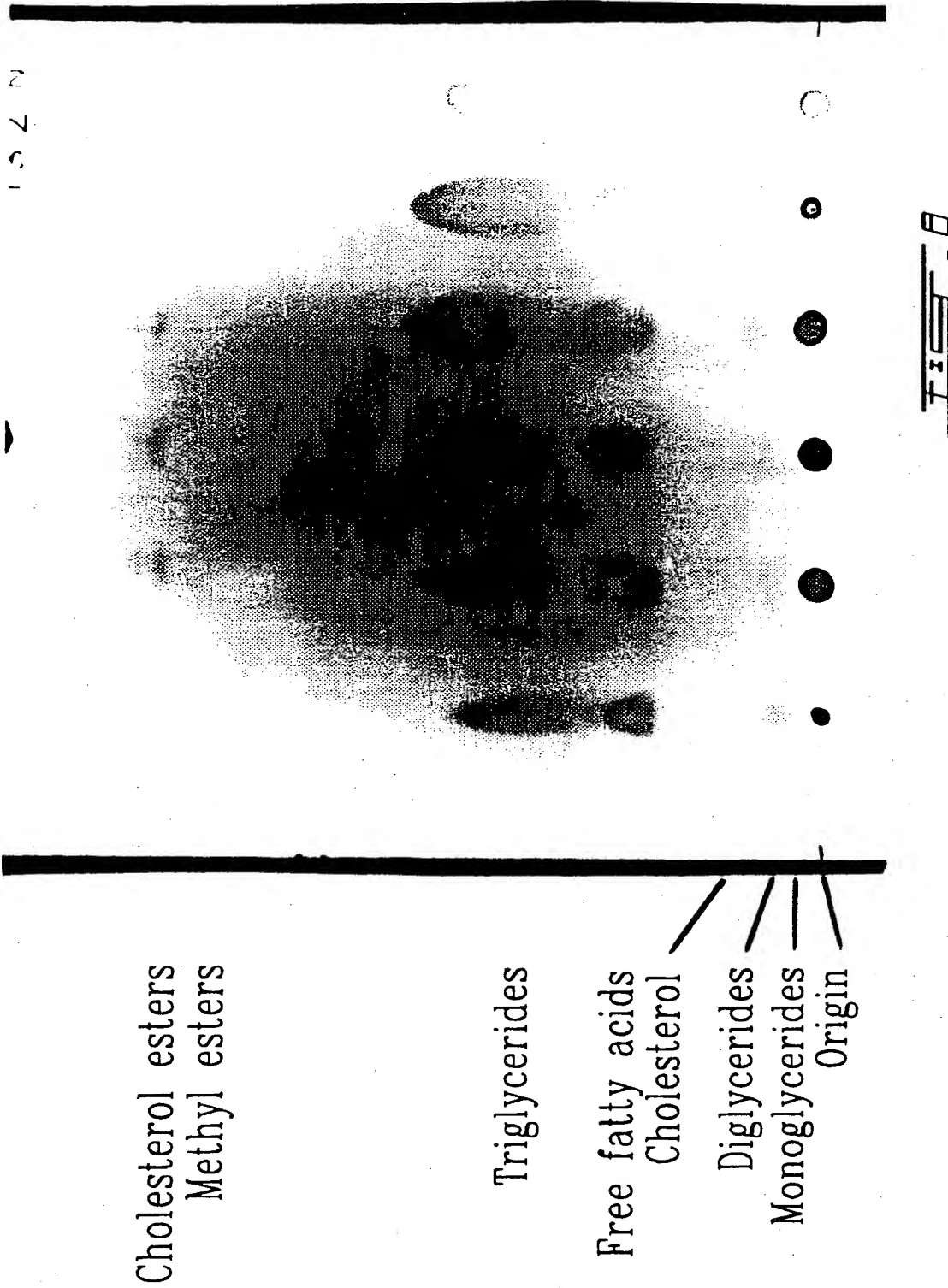
1.564 - 12:0	8.025	34.677
1.762	8.485	38.373 - 22:0
1.977	9.360 - 18:0	40.163
2.080	9.407	50.157
2.273	10.147 - 18:1tr	57.532
2.591 - 14:0	11.618 - 18:2	61.436
2.887 - 14:1	12.858	70.271
3.008	14.515 - 18:3	77.784 - 24:0
3.470 - std 15:0	16.162	110.694
4.108	18.077	127.696
4.341	19.355 - 20:1	
4.803 - 16:0	20.182 - 20:4 (cis11)	
5.210 - 16:1tr	20.311	
5.683	23.205 - 20:2	
6.292	24.678	
6.514	27.411 - 20:4 (6,10,14,18)	
7.030	29.307	
7.810	31.990	



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Cholesterol esters  
Methyl esters

Triglycerides

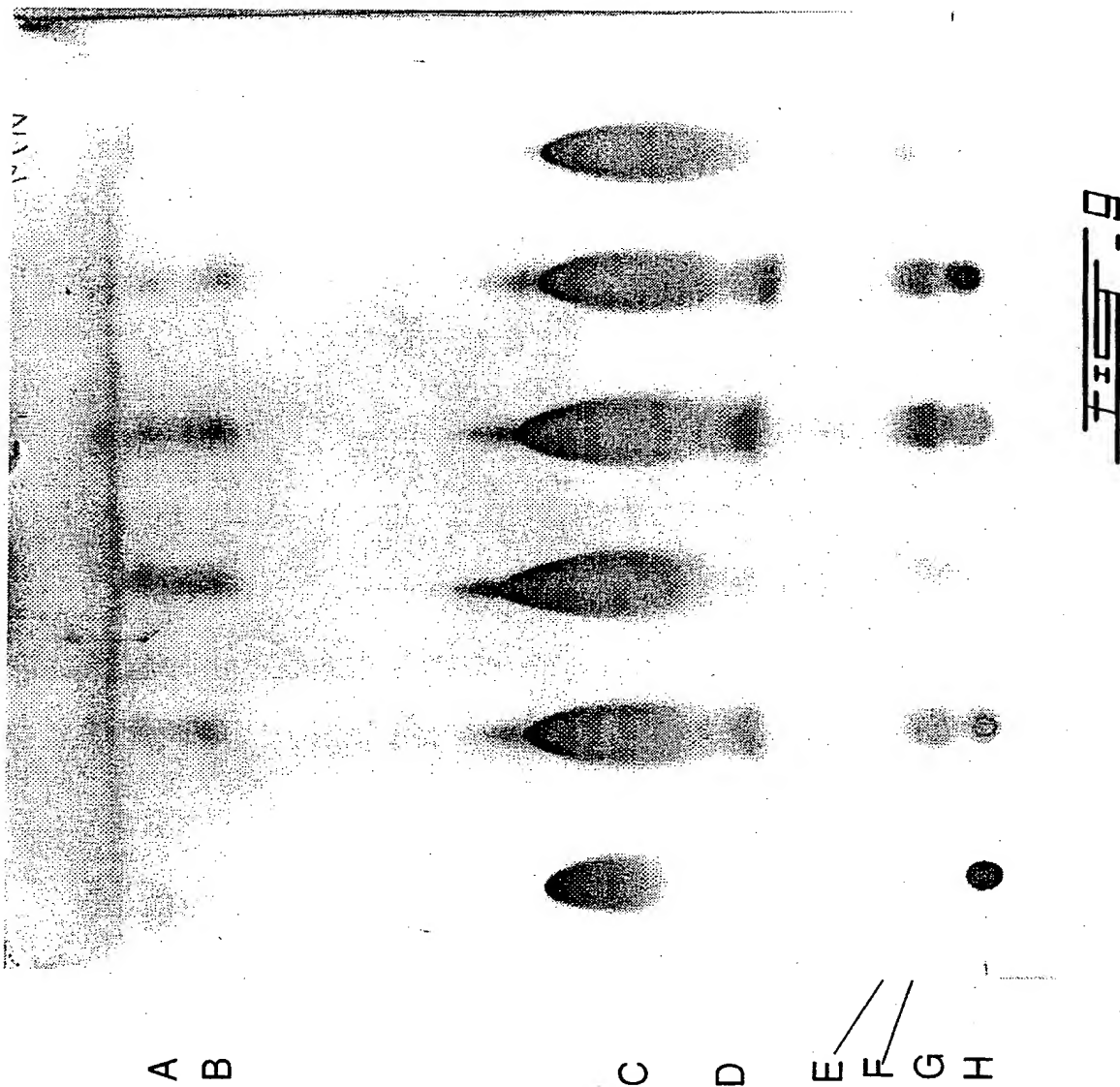
Free fatty acids  
Cholesterol

Diglycerides

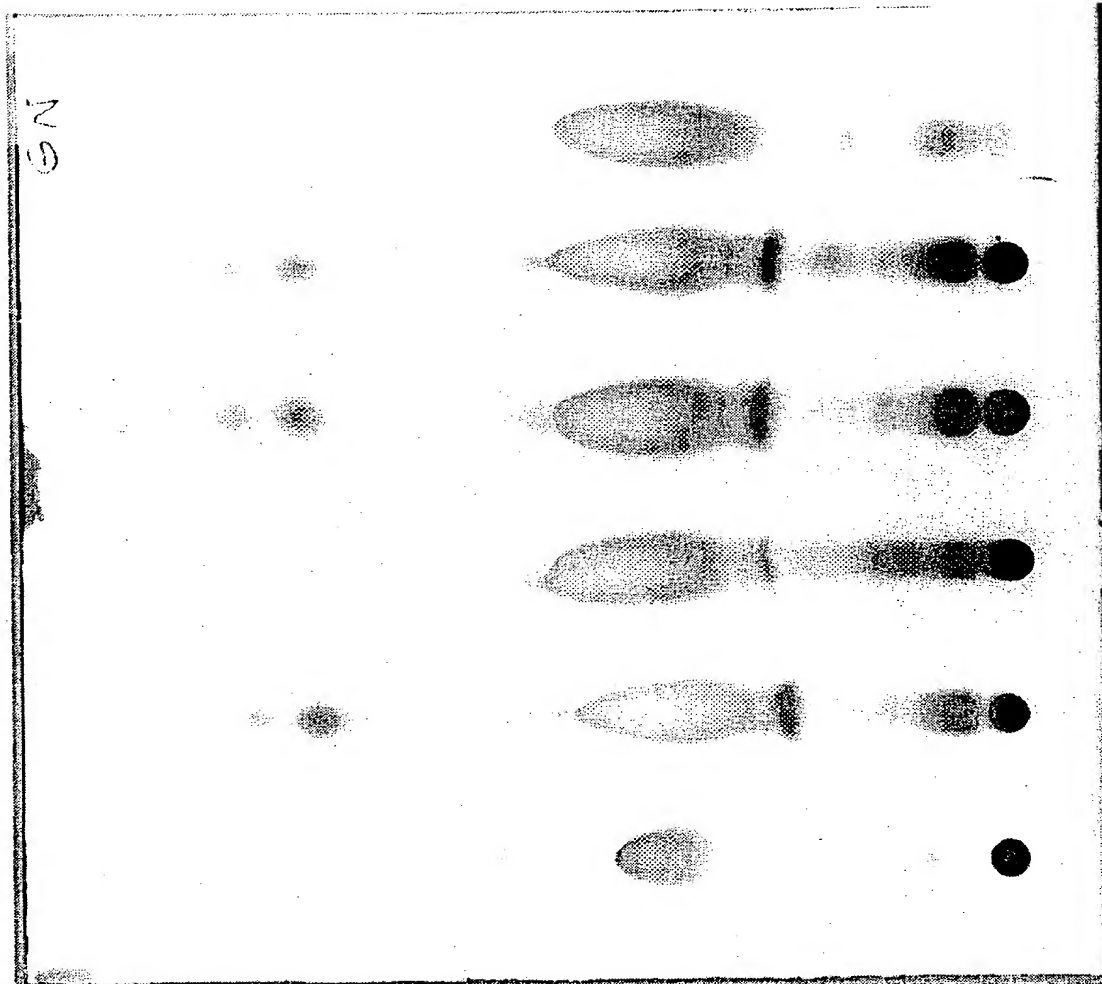
Monoglycerides

Origin

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A

B

C

D

E

F

G

H

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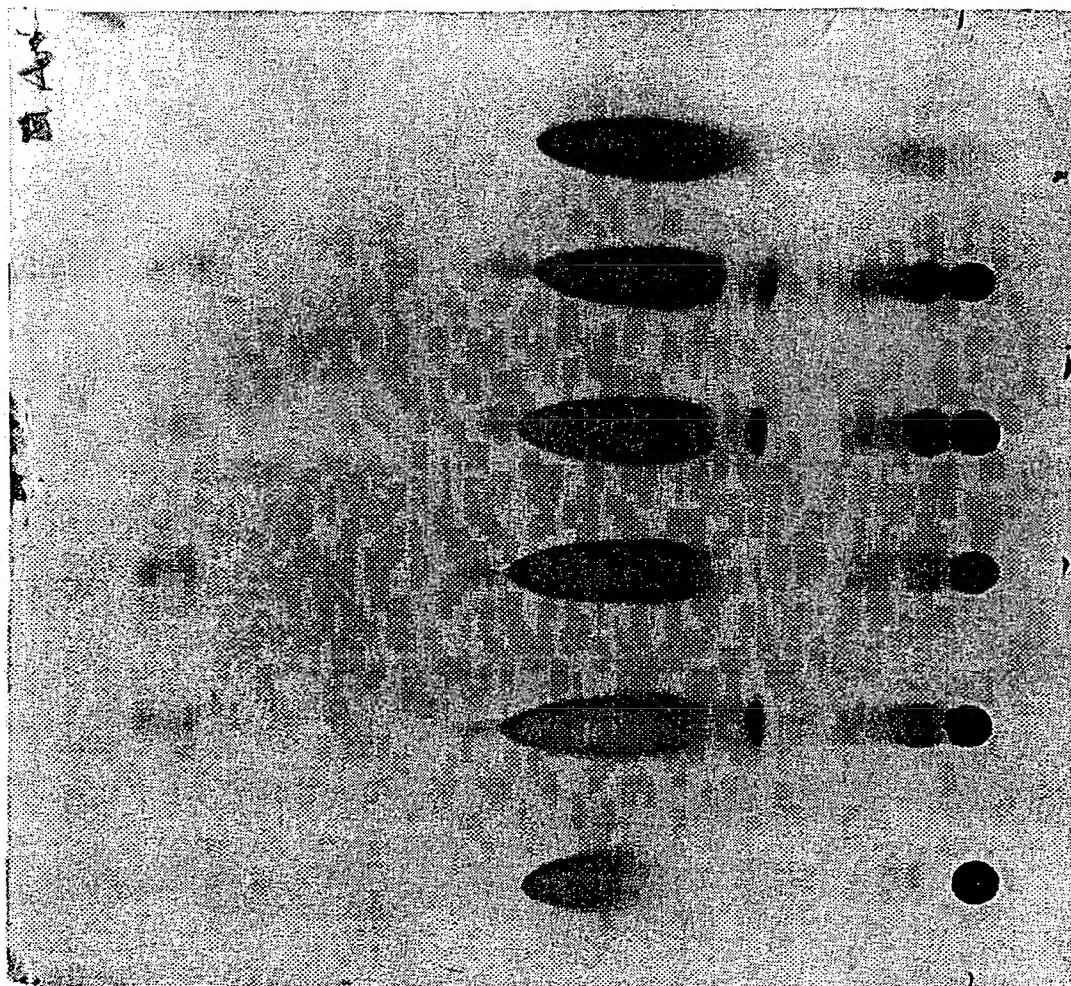


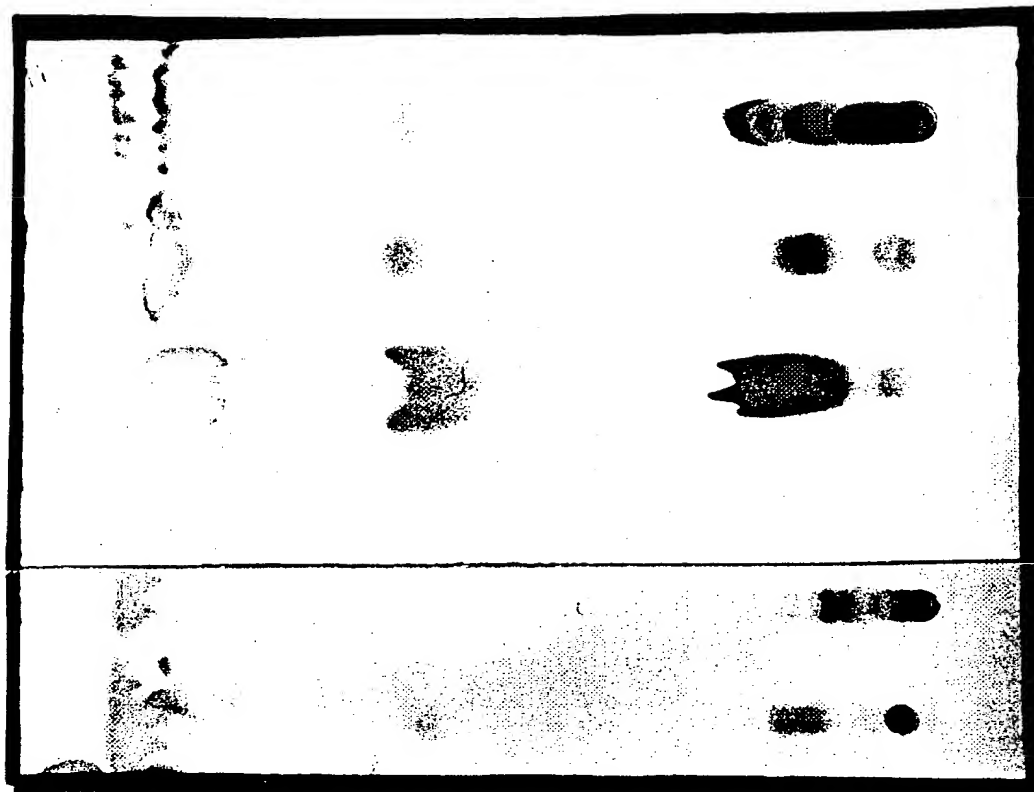
Fig. 11

A B

C

D E F G H

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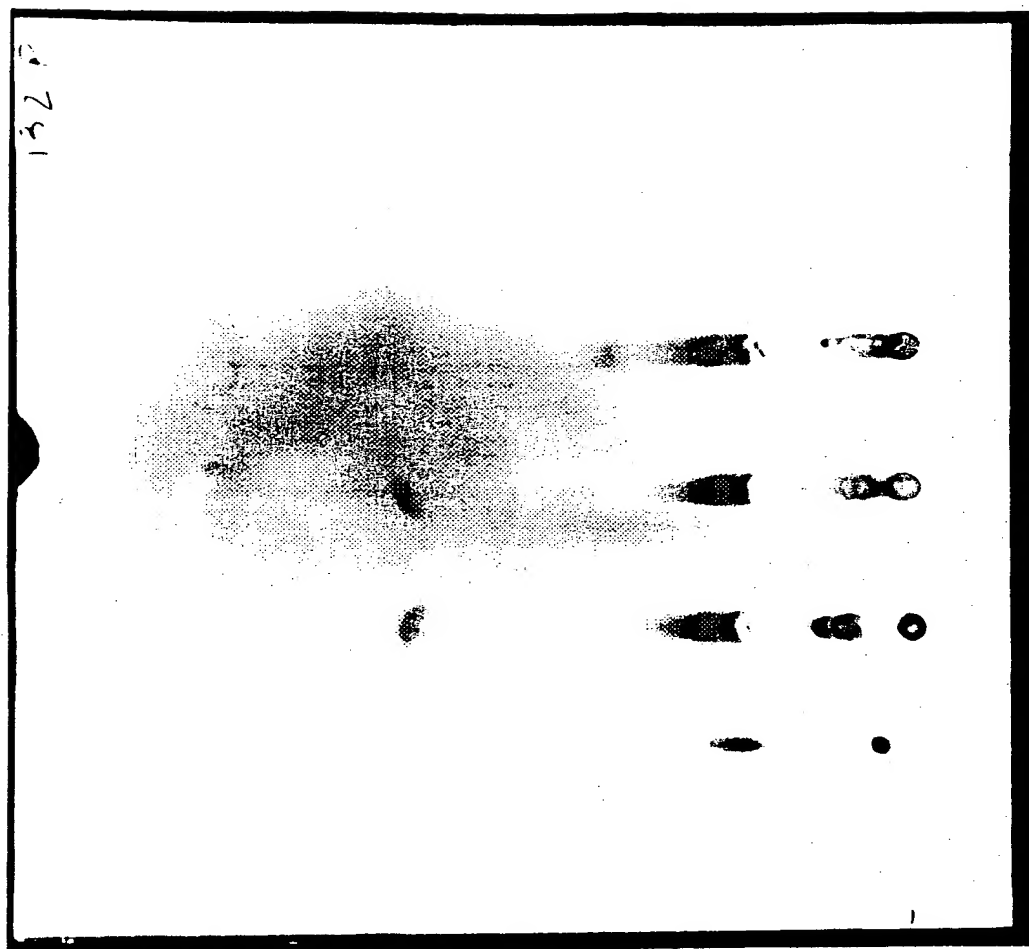
Neutral lipids

Cephalin

Lecithin  
Sphingomyelin  
Lysolecithin  
Origin

12-12

13/20



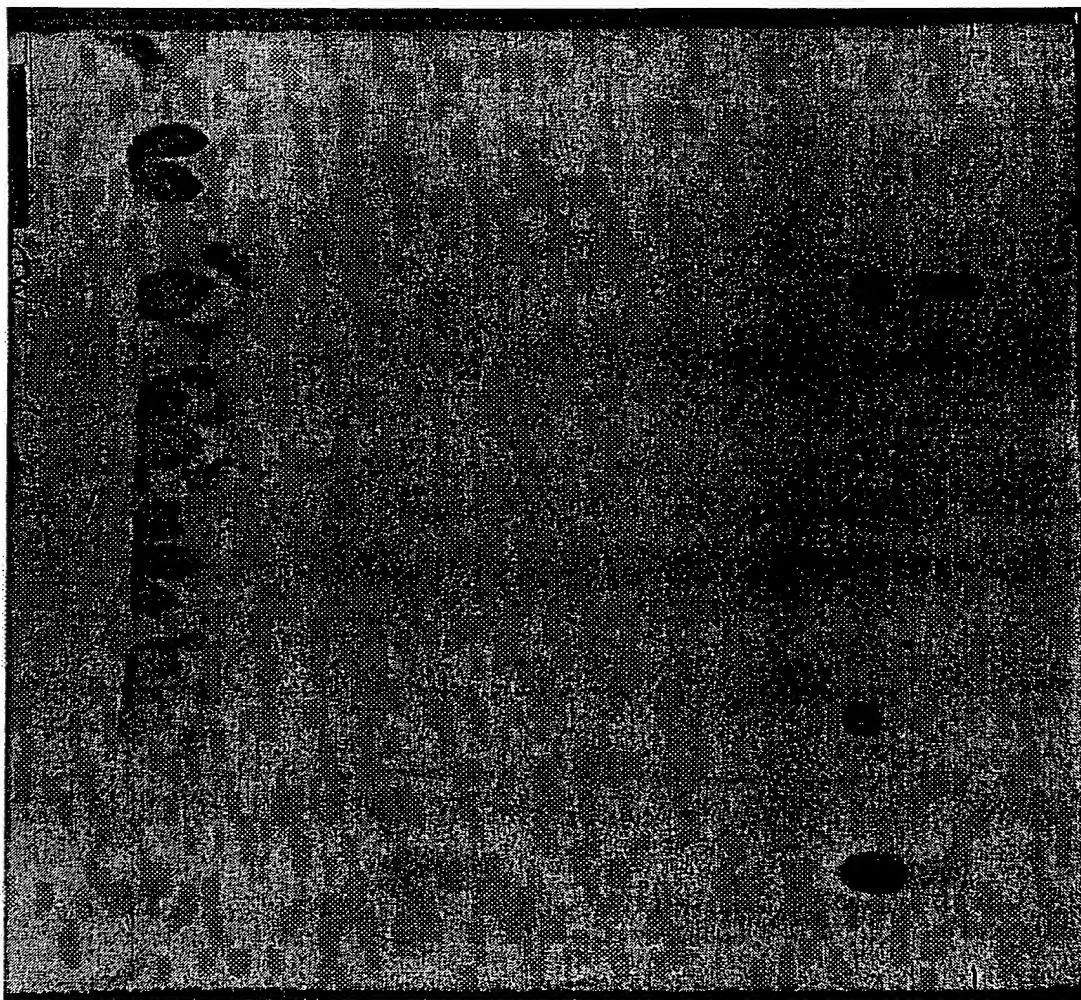
Neutral lipids

Cephalin

Lecithin  
Sphingomyelin  
Lysolecithin  
Origin

132-13

14/20



FILE - 14

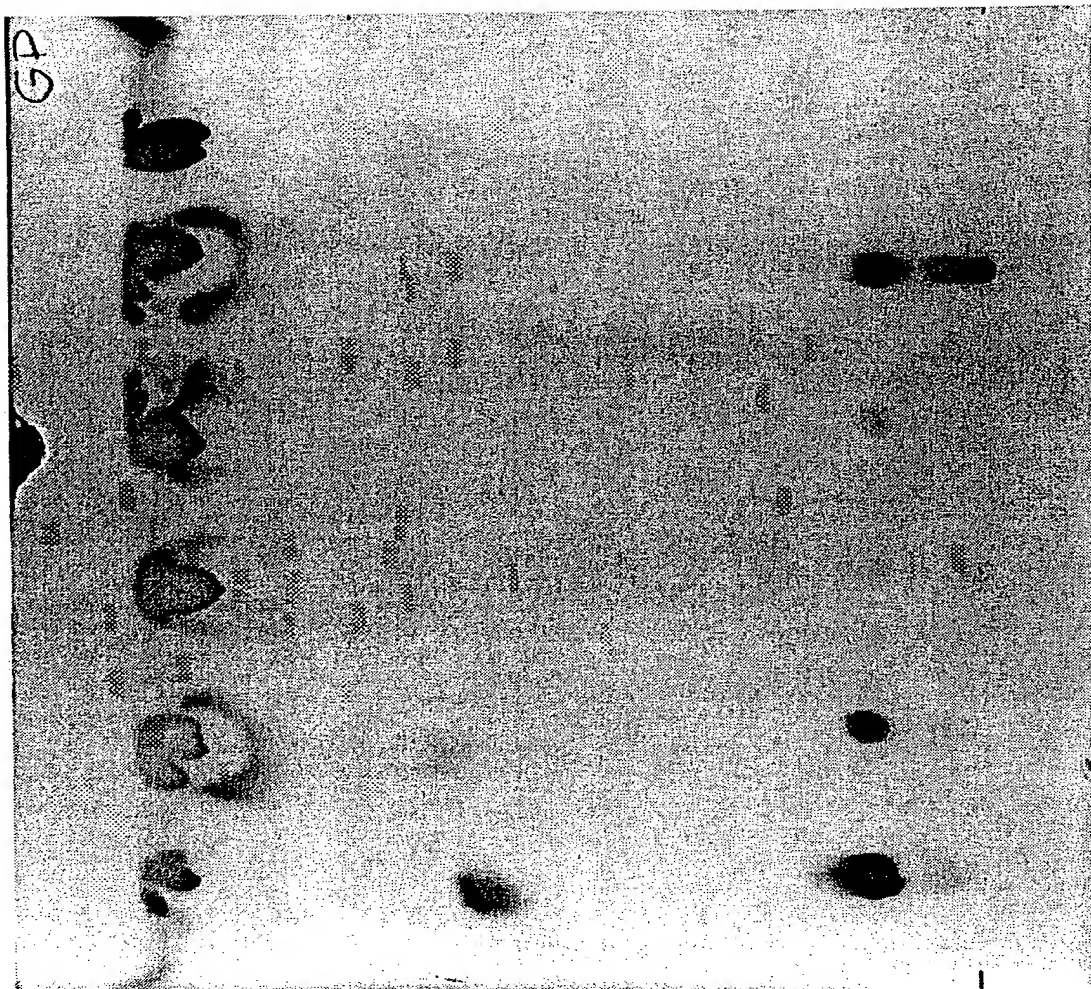
A

B

//  
//  
//  
O D E F



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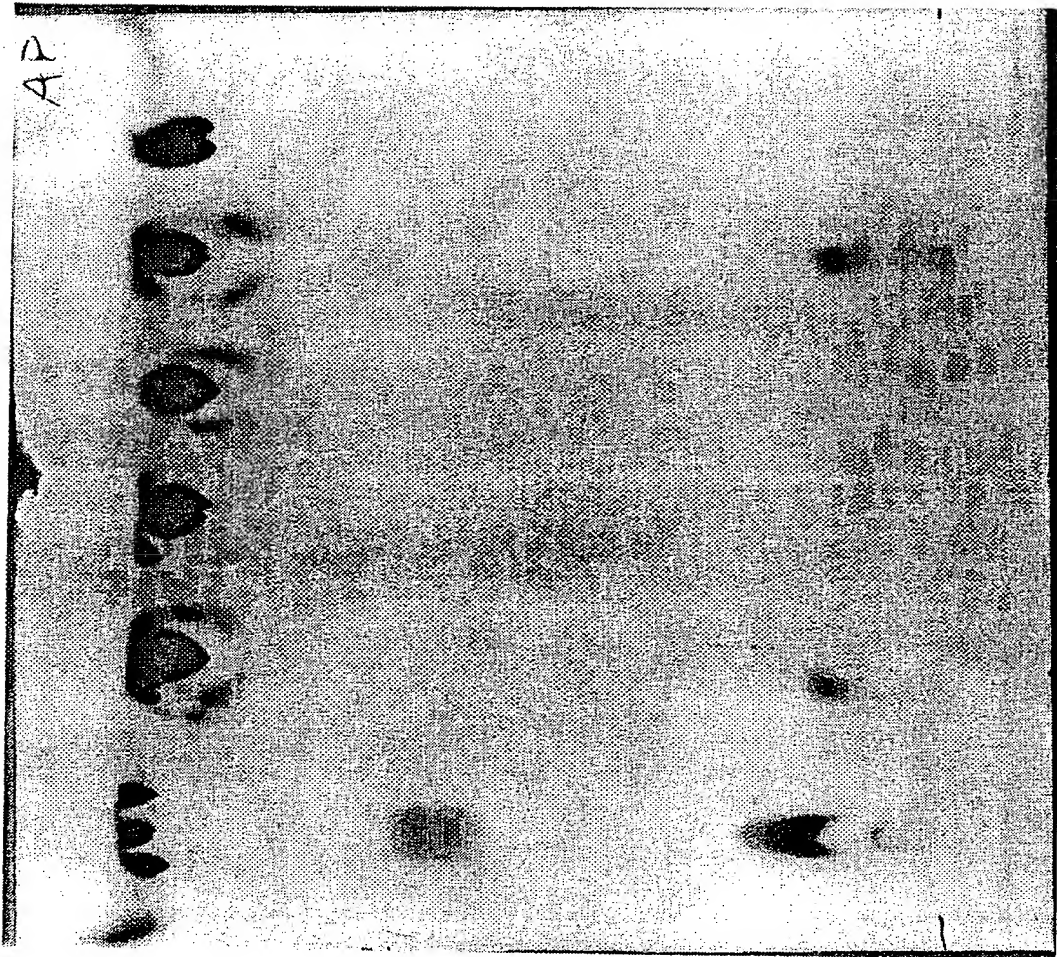


A

B

///  
C D E F

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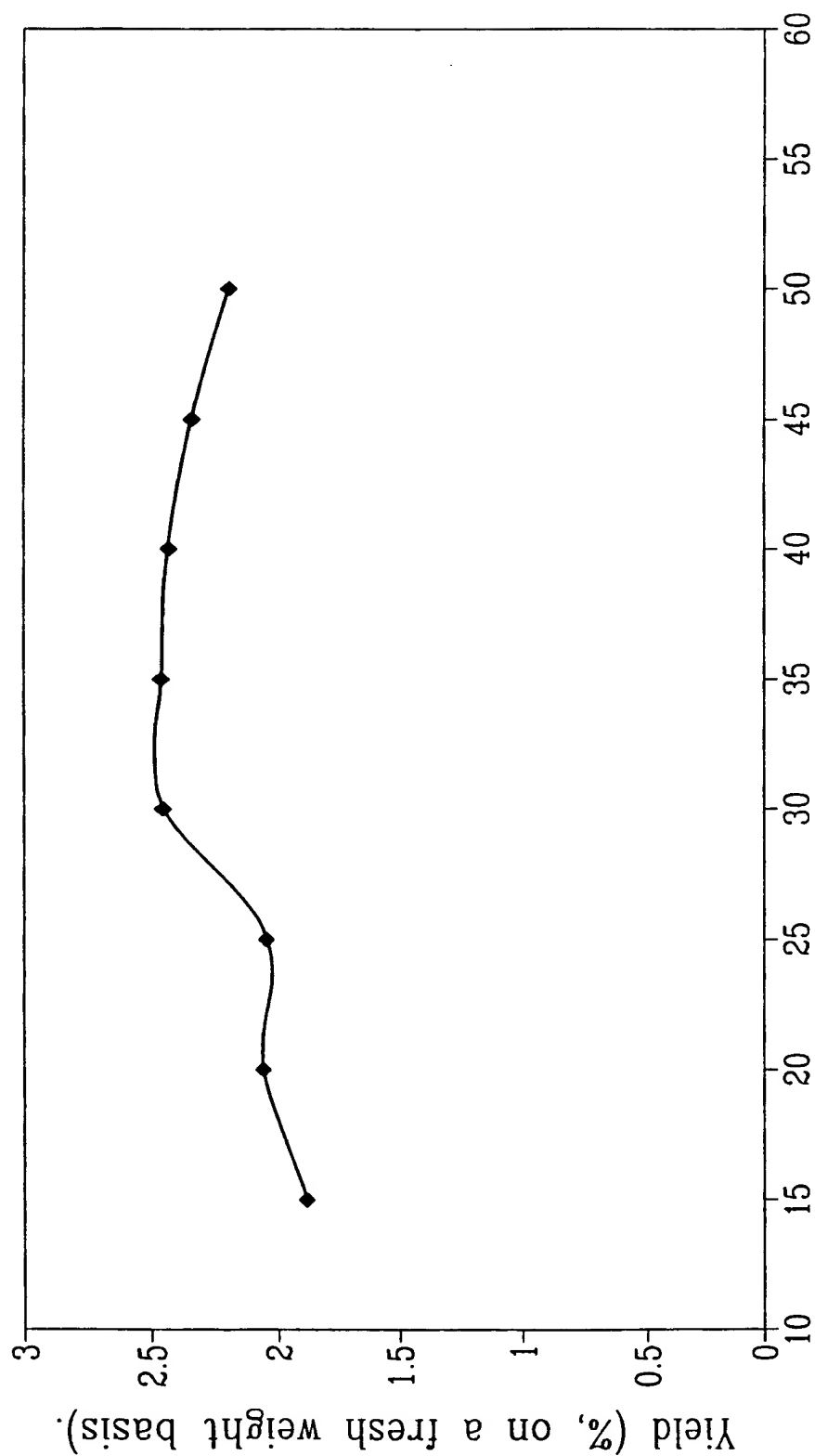


A

B

C D E F

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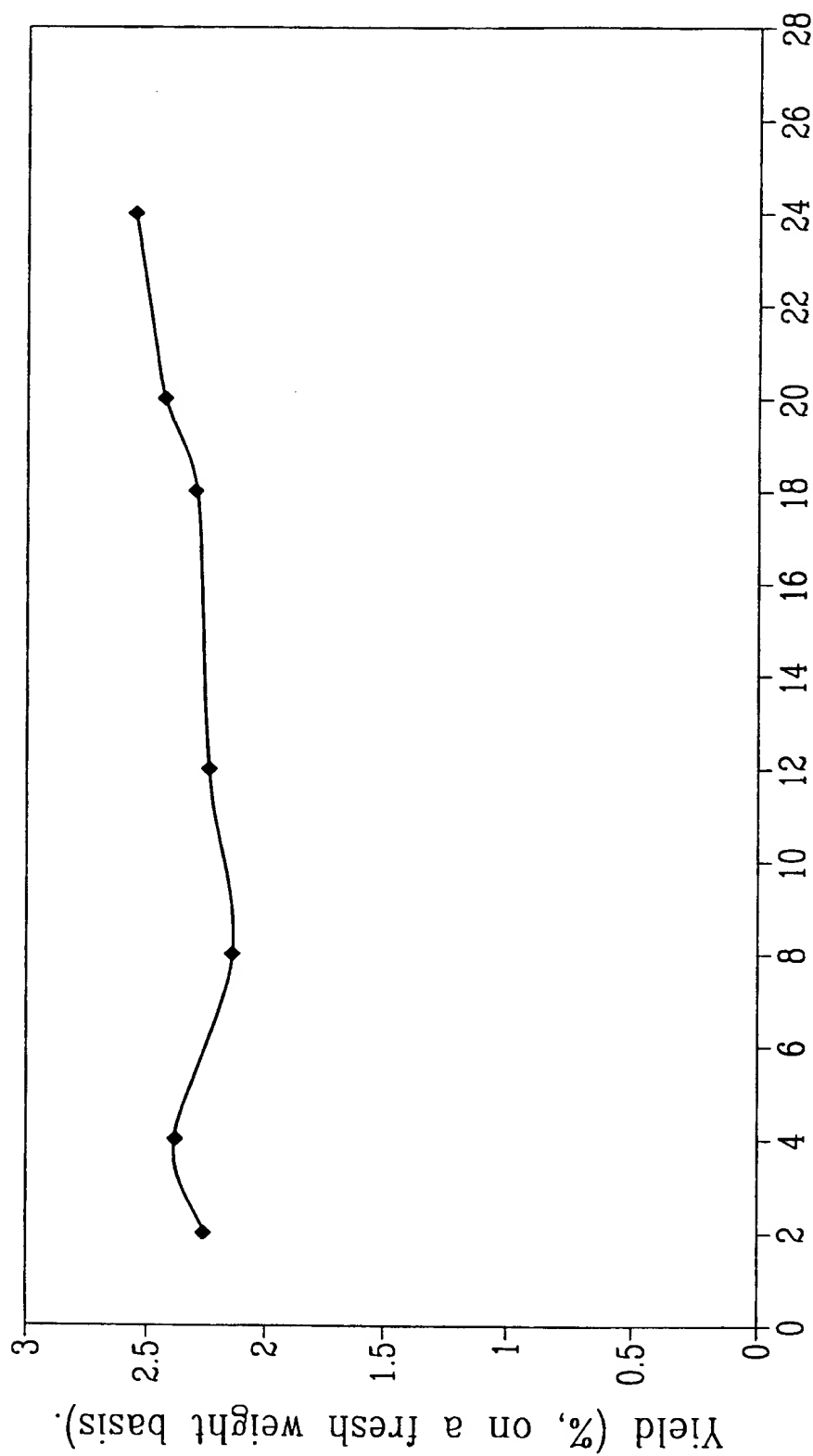
Volume of acetone (mL).

Incubation time of 2 h.

Determinations in triplicates (variation less than 5 %).

FILE - 17

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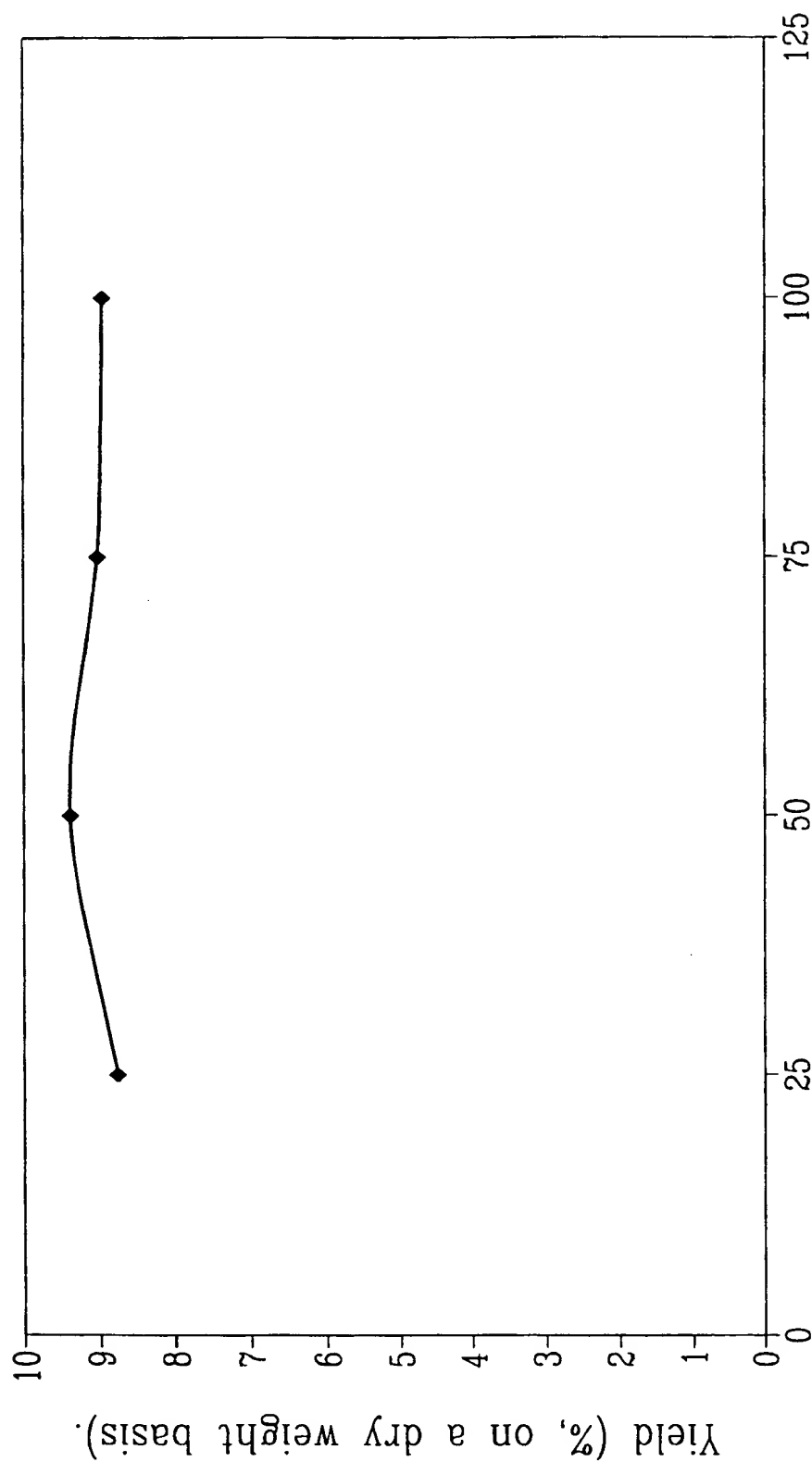
Incubation time in acetone (h).

Sample-acetone ratio of 1:9 (w/v).

Determinations in triplicates (variation less than 5 %).

FIG-18

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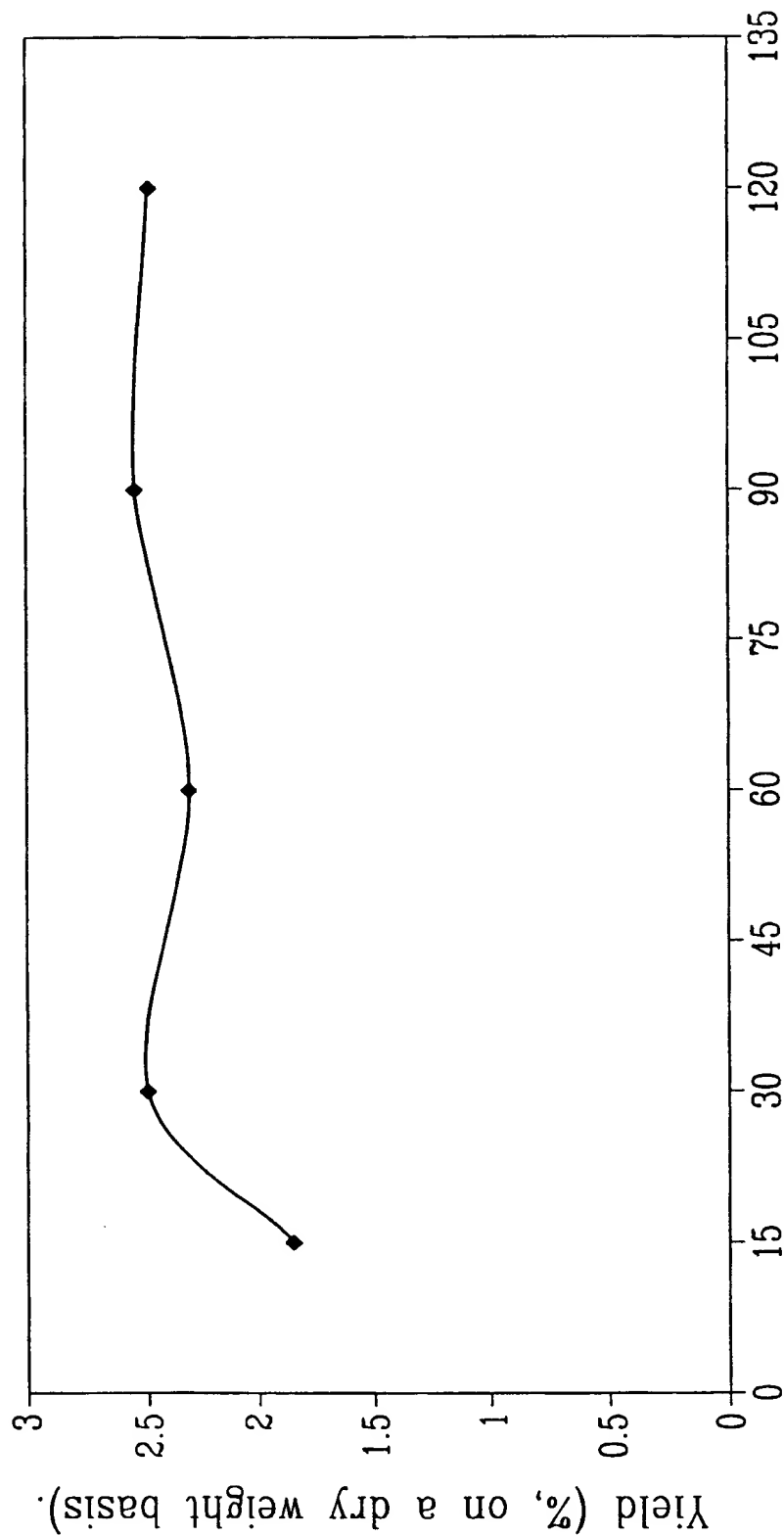
Volume of ethanol (mL).

Incubation time of 30 min.

Determinations in triplicates (variation less than 5 %).

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Incubation time in ethanol (min).

Sample-ethanol ratio of 1:4 (w/v).

Determinations in triplicates (variation less than 5 %).

FIG - 20